Minimal Media

Notebook:	iGEM 2014		
Created:	7/3/2014 11:59 AM	**Updated:**	10/14/2014 11:45 PM
Author:	yaminitn	**Location:**	41°15'20 N 72°59'22 W

M9 Salts (1X) requires 200 mL per 1L Minimal media
MgSO4 (2 mM) requires 2 mL per 1 L Minimal Media
☐ 36.1 mg of MgSO4 to 150 mL of dH20
Glucose (0.4% wt/vol) 45 mL per 1 L Minimal Media
Biotin (0.25 micrograms/microliter) 1 mL per 1 L Minimal Media
Thiamine (0.00005% wt/vol) 100 microliters per 1 L Minimal Media
CaCl2 (0.1 mM) 100 microliters per 1 L Minimal Media. Always add last.

TO DO (Research)

Author: stephanie_mao

1. Riboregulation System				
Task	**Approx. Time**	**Due Date**	**Handled by**	**Status**
Order sequencing primers			Stephanie	Ordered
Amplify insert				
AGE amplicons				
PCR purify amplicans				
Send to Keck for sequencing				

Parts needed: Sequencing primers (ordered from Keck + 810S, 39S and 811S stored in box labelled "T7 RR" in small fridge) Primer pairs: 810+ seq_1r, seq_2f+39S, seq_3f+seq_3r, seq_4f+811S
Plasmid: A12C miniprep labelled with most recent date, in same box as primers. Or, use the most recently streaked ECNR2+pZE21_A12C_T7RNApol streaked on Kan (half plate, other half is control)

In parallel: remaking the construct from scratch and doing a functional assay BEFORE sequencing.
Primers: 191 and 192 for T7 (58˚C annealing, 2:30 extension time) and 195 and 196 for the backbone (Two-step 58˚C and 68˚C, 2:30 ext. time)

- PCR ☑
- DPN1 digest ☑
- Gel purify ☑
- Drop Dialysis ☑
- Transform into ECNR2+pZA21_T7sfGFP (incubating 9/7) ☑
- Plate on Kan+Spe+ATC+IPTG
- Do a FACS selection on colonies that are fluorescing (shows that T7 is functional) ☑
    - Plate FACS selection on Kan+Spe ☑
    - Screening:
        - Colonies picked and growing in incubator (9/19, someone should take it out on 9/20) REDO
        - sequencing: use 810+T7_1f since we want mostly the riboregulation system. ☑
            - PCR purify + gel ☐
            -
        - Functional: grow it up w/ and w/out inducers and do a plate reader to compare fluorescence. ☐
- Grow up colony in YT and perform a mini prep. ☐
- Transform into Mach1 and plate on ONLY Kan. The pZA21 plasmid should be lost ☐

2. Antimicrobial Peptide				
Task	**Approx. Time**	**Due Date**	**Handled by**	**Status**
Design Primers for Gibson			Stephanie	Ordered
Gibson Construct into PZE21				
Transform OTS and peptide plasmid in				
Integrate ATC-T7RNA into RK24				on hold
Integrate ATC-T7RNA into genome				on hold
2.5 Extraction				
Induce with arabinose & ATC (select with chl and kan)				
Order twin strep extraction			Natalie	Done
Test twin strep extraction on normal HisTag				on hold
Twin Strep extraction of our construct				
Made up metal extraction thingamabob				

Parts needed: recoded strain 730 streaked in fridge. Construct transformed into DH5A, can use pZE plasmid backbone from failed T7 RNA pol construct (look for plates with "INCORRECT" written on them)

3. Peptide Assays				
Task	**Approx. Time**	**Due Date**	**Handled by**	**Status**
Anchorage assay test (contact angle)				on hold
Adhesive modeling				
Biofilm assay control (with standard antibiotic)				

T7-RiboRegulation System

- [x] Primers from Keck arrived and diluted - Ariel
- [x] Meet With Dr. Isaacs at 3 PM.
- [x] Sequencing reactions prepped and sent to Keck

Interlab - Yamini is Boss.
Should the transformation be successful on 8/28, we just need to devote around 1.5 hours a day for two days to run a GFP assay. Plates are in the fridge downstairs labelled "DH5A Interlab X" with chloramphenicol resistance (plate labelled either red or purple)

- [x] Interlab needs to be transformed.
- [x] Start liquid culture of DH5-alpha.
- [x] Find Plasmid for Interlab Transformation 1 from plates. (Second Source)
- [x] Transform into DH5-alpha. Plate on Kanamycin (Double check this plasmid).
  Second attempt 9/7: check incubator. SUCCESSFUL
- [x] 9/9/14: Growing up 3 150 µL of each Interlab strain, seeded from same colony in Kan (Interlab 1) and Cam (Interlab 2 and 3). Will conduct a GFP assay tomorrow.

Preparing Construct for transfer to Expression Vector: Ariel

- [x] Transformed GenScript construct into DH5alpha. Colonies grew succesfully. Going to design primers to amplify and gibson assemble the constructs we want into pze vector backbone. - Ariel
- [x] Starting liquid culture of construct and of cells with pze21:sfgfp for pcr amplification of backbone. -Ariel
- [x] Need to design primers that individualize the two connected construct modules, the MAP-GFP and the MAP-AMP. Currently only have primers that target external.
- [x] Miniprep cells containing construct and pze21 backbone
- [x] Pcr amplification of backbone pze21 and constructs. Primers 253 and 254 for the construct. Primers 255 and 256 for pze21 backbone. Verify sequences.
- [x] Pcr runs overnight. Needs to be picked up. Also started cultures of mach1, construct, and pzecat for tomorrow just in case.
- [x] DPN1 digest of PCR amplicons
- [x] Make SYBR Green Gel
- [x] Run PCR fragments on a SYBR green gel
- [x] Gel purify fragments.
- [x] Gibson Assemble Fragments.
- [x] Drop Dialysis of Gibson Assembly Product
- [x] Transform Gibson Assembly Product into Mach1, ECNR2, and C3PO
  C3PO transformed successfully, selected 11 colonies for screening.
  12 cultures grew for too long, selected another 11 alongside a dilution of the first 12. Will screen on 9/10/14.
- [x] Screen 96 well plate of colonies with primers 810 & 811.
- [x] Grow Up Successful Colonies in YT. And lb
- [x] Make frozen stock of 730: gen-script plasmid that works.
- [x] [x] Make Primers for Separating Two Constructs. Make Screening Primers.
- [x] Mini-Prep for plasmid.
- [ ] Second Mini-prep of Plasmid - For back-up.
  - [ ] Assemble MAP-GFP Plasmid
    Primers were made for the excision of sfGFP from the construct. The forward site anneals to the end of sfGFP sequence and amplifies the backbone. The reverse primer anneals just before the beginning of the sfGFP plasmid. It amplifies the backbone. Each adds on a Not1 site for restriction digest.
    - [x] Amplify Miniprepped construct_pZE21 with GFP Excision primers.
    - [x] DPN1 Digest Fragments
    - [x] PCR purify fragments. Run 5 uL on gel to verify correct size.
    - [x] Conduct Not1 Digest of the amplicon.
    - [x] PCR purify the digested fragments. Conduct T4 Ligation reaction and transform into Strain 730.
    - [ ] Screening can be performed with primers 811 and 49.
  - [ ] Assemble GFP-MAP Plasmid
    Modular Restriction site is already present around the MAP.
    - [x] Restriction digest mini-prepped plasmid with KPN1.
    - [x] Conduct PCR purification.
    - [ ] Run T4 Ligation Reaction and Transform into 730
    - [ ] Screening can be performed with primer 810 and 50.

- [ ] Express as whole construct in pZE21 plasmid. At Beginning and At MidLog. Ask Mira about what conditions she uses for DOPA expression.
- [ ] Express as GFP-MAP in pZE21 plasmid
- [ ] Express as AMP-MAP in pZE21 plasmid.

TO DO (Fall Semester Edition)

Author: stephanie_mao

1. Riboregulation System				

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Send to Keck for sequencing				

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Plasmid: A12C miniprep labelled with most recent date, in same box as primers. Or, use the most recently streaked ECNR2+pZE21_A12C_T7RNApol streaked on Kan (half plate, other half is control)

- [x] Primers from Keck arrived and diluted - Ariel
- [ ]

2. Antimicrobial Peptide				
Task	**Approx. Time**	**Due Date**	**Handled by**	**Status**
Design Primers for Gibson			Stephanie	Ordered
Gibson Construct into PZE21				
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Integrate ATC-T7RNA into RK24				on hold
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Induce with arabinose & ATC (select with chl and kan)				
Order twin strep extraction			Natalie	Done
Test twin strep extraction on normal HisTag				on hold
Twin Strep extraction of our construct				
Made up metal extraction thingamabob				

Parts needed: recoded strain 730 streaked in fridge. Construct transformed into DH5A, can use pZE plasmid backbone from failed T7 RNA pol construct (look for plates with "INCORRECT" written on them)

3. Peptide Assays				
Task	**Approx. Time**	**Due Date**	**Handled by**	**Status**
Anchorage assay test (contact angle)			Alex	In Progress (Collaboration with Dufresne Lab)
Optical Tweezer Assay			Alex	In Progress (Collaboration with Zhang Lab)
Adhesive modeling				
Biofilm assay control (with standard antibiotic)				

Interlab
Should the transformation be successful on 8/28, we just need to devote around 1.5 hours a day for two days to run a GFP assay. Plates are in the fridge downstairs labelled "DH5A Interlab X" with chloramphenicol resistance (plate labelled either red or purple)

- [ ] Interlab transformation was not succesful. (8/29) Will need to be redone next week on monday. -Ariel

Preparing Construct for transfer to Expression Vector

- [x] Transformed GenScript construct into DH5alpha. Colonies grew succesfully. Going to design primers to amplify and gibson assemble the constructs we want into pze vector backbone. - Ariel
- [x] Starting liquid culture of construct and of cells with pze21:sfgfp for pcr amplification of backbone. -Ariel
- [x] Need to design primers that individualize the two connected construct modules, the MAP-GFP and the MAP-AMP. Currently only have primers that target external.
- [x] Miniprep cells containing construct and pze21 backbone
- [x] Pcr amplification of backbone pze21 and constructs. Primers 253 and 254 for the construct. Primers 255 and 256 for pze21 backbone. Verify sequences.
- [ ] Pcr runs overnight. Needs to be picked up. Also started cultures of mach1, construct, and pzecat for tomorrow just in case.
- [ ] DPN1 digest of PCR amplicons
- [ ] Run PCR fragments on a SYBR green gel
- [ ] Gel purify fragments.
- [ ]

Interlab Worksheet

Author: stephanie_mao

Section I: Provenance and Release

1. The Interlab measurement study was conducted by Ariel Leyva-Hernandez, Yamini Naidu and Stephanie Mao.
2. The study was conducted in Dr. Farren Isaacs' lab, with the mentoring of Natalie Ma.
3. The construct protocols were finalized at the end of June, and the constructs were made in the span of July 21-September 3. The measurements were taken on September 9, 2014, and again on September 12, 2014.
4. All persons mentioned give consent for their names to be included in the study.

Section II: Protocol

1. Devices 2 and 3 were made using PCR with the specific promoter region in the forward and reverse overhangs, and BBa_E0240 as the template. The DNA was then DpnI digested and self-annealed to itself using a Gibson Assembly mix. This device was transformed into the strain Mach1, and colonies were screened before a plasmid miniprep was transformed into DH5A.
2. Measurements were conducted on the Synergy H1 Hybrid Multi-Mode Microplate Reader manufactured by Biotek. It is configured for Monochromator fluorescence, Monochromator absorbance,Full-light luminescence,Time resolved fluorescence,Temperature control to 45°C, and Gen5 data analysis software.
3.

Section III: Measured Quantities

Section IV: Measurements

T7 Riboregulator Verification (Screening)

Author: M. Chen

1. Riboregulation System				
Task	**Approx. Time**	**Due Date**	**Handled by**	**Status**
Order sequencing primers			Stephanie	Ordered
Amplify insert				
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PCR purify amplicans				
Send to Keck for sequencing				

Parts needed: Sequencing primers (ordered from Keck + 810S, 39S and 811S stored in box labelled “T7 RR” in small fridge) Primer pairs: 810+ seq_1r-fixed, seq_2f+39S, seq_3f+seq_3r, seq_4f+811S
Plasmid: A12C miniprep labelled with most recent date, in same box as primers. Or, use the most recently streaked ECNR2+pZE21_A12C_T7RNApol streaked on Kan (half plate, other half is control)
☑ Primers from Keck arrived and diluted - Ariel

Reaction 1:
Primer 810 (f) [55C]
Primer Seq1R-fixed (r) [59C]

Reaction 2:
Seq 2F (f) [59C]
39S (r) [53C]

Reaction 3:
Seq 3F (f) [58C]
Seq 3R (r) [58C]

Reaction 4:
Seq 4F (f) [58C]
811S (r) [54C]

A12C miniprep;

200 ng/uL; dilute 10 into 90;
one degree lower than lower one
Primer Seq1R-fixed, Seq2F, Seq3F, Seq3R, Seq4F diluted by a factor of ten.

1 uL of each primer
1 uL of DNA (verified)
22 uL water
25 uL readymix

Reactions 1, 2 and 4 ran on thermocycler (1 minute extension time, 52 degrees annealing temperature) in block A

Reaction 3 (1 minute extension time, 57 degrees annealing in block B)

Products DNA purified;

Ran on gel; image is on GelDoc; Bands appear to be correct

Sequencing reactions set up, Keck orders made.

It looks...kind of unsalvageable

1.5 grams of agarose;
150 TBE
cyber green 15 uL;

Lab Clean-Up Work

Lab Clean-Up Work

Map
Author: Ariel Hernandez-Leyva
- [x] Take Out Trash (Serious Business... Autoclave it).
- [x] 3 Bottle LB min + 1 Bottle YT.
- [ ] Bleach The CounterTops.
- [x] Organize Primers, Plasmids, and Sequencing Primers.
- [ ] Go through Strain Box and Verify Database in Dry Ice.
- [ ] Make up a new chloramphenicol. Test chloramphenicol against Natalie's.
- [ ] Take out the damn trash. And again. And always.

People:
Ming, Ben, Alex, Stephanie, Ariel

1st Priority:
- [ ] Interlab

BioBricks 2014

Map
Author: Ariel Hernandez-Leyva
- [ ] DOPA Orthogonal Translation Systems
- [ ] Tryrosine Supressor
- [ ] T7-sfGFP
- [ ] T7 RNA Pol under pLtetO
- [ ] MFP-GFP
- [ ] AMP-MFP

TO DO

Author: stephanie_mao

July 18, 2014

- Autoclave

- [x]

- Transformation of T7-GFP-pZA15

- [x]

  - need to wait until agar plates are made with spec
  - Plate it

- [x]

- Make more culture of Mach1 and interlab alpha culture-yamini

- [x]

- Start a Yt culture of T7 Promoter

- [x]

- Mini-prep T7 RNA pol samples (11), Nanodrop plasmid concentrations.

- [x]

- Run restriction enzyme digest (1 hour protocol, cut buffer) with KPN1 and HindIII and run gel.

- [x]

- Run gel on massive PCR done with Ryan's sequencing primers/Internal primers combination.
- (Should the primers get here) Get T7+sfGFP into pZA21

- [x]

- Redo Interlab study

- [ ]

  - Kappa Fast PCR with 197/199 for promoter 3, and 198/200 for promoter 2

- [x]

  - DPN1 digest

- [x]

  - Run gel to test correct fragment length

- [x]

  - Redo 3

- Gibson assemble.

- [ ]

- Drop dialyze

- [ ]

- Transform into DSH alpha

- [ ]

- Keep on looking up respective areas (Stephanie-constructs, Yamini-Biofilm assays, Ariel-Extraction protocol)

- [x]

- Finalizing Polymer Design- Yamini

- [x]

- Put away the Kan in freezer before leaving the lab for the day

- [x]

July 21, 2014

- Kappa Fast PCR with 219/220 for T7+sfGFP, and 221/222 for pZA21 (mini prep plasmid)

- [ ]

    - 220 wrong, forgot that I'm keeping in a bit of the old GFP, reordered primer.

- DPN1 digest

- [ ]

- Gel purify

- [ ]

- Gibson assemble

- [ ]

- Drop dialyze

- [ ]

- Transform into Mach 1

- [ ]

- Miniprep culture that I started but completely forgot what the heck it was, sorry (Stephanie) (On bench)

- [x]

- Check pZA21 plates on bench.

- [ ]

- Redo Interlab study

- [x]

    - May have to re-transform plasmid if I don't have a strain with just that plasmid, although I recall someone doing that transformation earlier. Maybe it failed...
        - Update: has strain, doing PCR from colony and starting culture for miniprep
    - Kappa HiFi Two-step PCR with 197/199 for promoter 3, and 198/200 for promoter 2 (use cell from frozen stock)

- [x]

- DPN1 digest

- [x]

- Run gel to test correct fragment length

- [x]

- Redo 3
- Gibson assemble.

- [x]

- Drop dialyze

- [x]

- Transform into DSH alpha

- [ ]

- Edit constructs to get rid of repeating sequence

- [x]
- Spec plates for pZA transformation, more agar just in case (we may need Kan+CAT, or Kan+IPTG, etc.

- [ ]
- Design primers to extract constructs when they get here.

- [x]
- Get DOPA OTS construct from Mira

- [x]
    - or we might have to make tyrosine OTS from the DOPA OTS

- [ ]
- Send original primer designs and sequences to Dr. Isaacs

- [ ]
    - Ariel planning to meet with Dr. Isaacs at 3:15
- Get oriented directly with Genscript contact, no middlemen

- [x]
- Cut plasmid with just XHO1 and Kpn1, or do a double digest to see if we get 2 bands

- [ ]
- Ask Jaymin about sequences we got from and then do a digest

- [ ]
- Look at DNA 2.0: find a good algorithm to optimize expression. Ask them if they can run it through their optimization system

- [x]
    - tell them we are iGEM students
- Ask Lexi for strain for tyrosine amber suppressor

- [ ]
    - or we can mutate with Mage oligos
    - use Mage to convert to Tyr Amber suppresor
- Check that P. Arginosa is BL1

- [x]
    - Yes it is BL1
- Look at more literature on biofilm assays

- [x]
    - Couldn't find any paper that uses MAPs and the MBEC assay
- More research on purification with tags
    - possible options:
    - Flag tag with MBP

- [ ]
    - Talk to Mira about ELP (using a salt gradient)

- [ ]
    - Is GST only on N-terminus?

- [x] AND C-Terminus
    - Dual step and flag tag - do a lit. search

- [x] This has happened.
    - see how people have used tags in combination, and look into whether N or C terminus specifics

- [ ]
- Set up with mtg with Dr. Isaacs tomorrow or Wed. to go into more detail on protein purification

- [ ]
    - Need to have a rough idea of top 2 tags
- T7_GFP:

- [ ]
    - Do with restriction enzymes
    - clean gene replacement

    - linear region just 16-18 base pairs
    - Try- our method, but also with restriction enzymes and quick-ligase protocol
- Look at NEB:

- [ ]
    - What bands look like on gel
    - resolving band lengths
- Get a recoded strain for rE.Coli OTS

- [ ]
- DOPA OTS system Miniprep out when we get it from Mira

- [ ]
- Make CAM Plates

- [ ]
- Look at Mira's ELP paper

- [ ]

July 22, 2014

- Conduct KPN1 digest of plasmid. Protocol included below. Continue cloning protocol for T7RR

- [ ]
    - Reaction Mixture
        - 35uL DNA from tube labeled for KPN1
        - 1 uL Enzyme
        - 4 uL Buffer
        - See Jaymin's protocol on sticky note in office to verify.
    - PCR purify post Digest. Now ready to Gibson Assemble.
- Fill water bath with water. Do not adjust temperature

- [ ]
- Order primers for restriction assembly of pZA21+T7sfGFP

- [x]
- Redo interlab 3 amplification and purification

- [x]
- Begin T7 promoter:sfgfp- pZA21 assembly with primers.

- [x]
- Begin new T7RR assembly protocol with primer 196.

- [x]
- Develop screening protocols for tomorrow. Will have a lot to screen.

- [ ]
- Make some fresh CAT

- [x]
- Prepare DOPA OTS and tyrosine supressor system

- [x]
- Look at NEB:

- [x]
    - What bands look like on gel
    - resolving band lengths
- T7_GFP:

- [ ]
    - Do with restriction enzymes
    - clean gene replacement
    - linear region just 16-18 base pairs
    - Try- our method, but also with restriction enzymes and quick-ligase protocol
- Set up with mtg with Dr. Isaacs tomorrow or Wed. to go into more detail on protein purification

- [x]
    - Need to have a rough idea of top 2 tags
    - Also go over our top 3 track selection
- More research on purification with tags- Yamini is working on
    - possible options:

- Flag tag with MBP

- [x]

- Talk to Mira about ELP (using a salt gradient)

- [x]

- Is GST only on N-terminus?

- [x] AND C-Terminus

- Dual strep and flag tag - do a lit. search

- [x] This has happened.

- see how people have used tags in combination, and look into whether N or C terminus specifics

- [x]

- Ask Jaymin about sequences we got from and then do a digest

- [x]

- Ariel: On the gel you're running, the T7sfGFP will be 786 bp, and the pZE tacr backbone will be 2600 bp.

July 23, 2014

- Check T7_pZE21 Plates Round 1 and 2 tomorrow

- [x]

- transformation of overnight drop dialysis of T7_pZE21 into Mach 1

- [ ]

- plate it on Kan plates

- [ ]

- Check overnight PCR of pZE21 tacr backbone in PCR 6

- [x]

- DPN1 digest

- [ ]

- Gel purify

- [ ] (fragment length of 2600 bp)

- Nanodrop

- [ ]

- Gibson assemble with T7 fragment

- [ ]

- Transform into Mach 1 strain

- [ ]

- Plate on Kan

- [ ]

- Gibson together T7sfGFP and pZA21

- [x]

- Transform into Mach 1 and successful T7pol+pZE21 if available.

- [ ]

- Transform Interlab into DS5 alpha

- [ ]

- Primers for the clean pZA21+T7sfGFP should arrive:
  - PCR amplify (225/226), too short for two-step

- [x]

- Gel purify

- [ ]

- Not enough pZA21 for restriction digest—inoculated 5 mL of YT with Mach 1 + pZA21
  - Restriction digest with AatII and BamHI+ligation

- [ ]

- Drop dialyze

- [ ]
    - Transform into successful T7pol+pZE21 and Mach 1

- [ ]
- Email Karl and Natash on SF-TAP protocol

- [x]
- Get P.Arginosa from Turner lab

- [ ]
- Put Gibson Ass. Mix cells in -80 degrees

- [x]
- Send Genotype information to Dr. Isaacs

- [x]
    - Make a streak so we have a frozen stock
- Protocol for LL-37 to Titanium

- [x]

July 24, 2014

- Constructing clean t7Promsfgfp_pZA21

- [ ]
    - Redo T7sfGFP PCR using HiFi, primers 225 and 226, with annealing temp at 54˚C and 45s extension time.

(Primers and template on Stephs bench)

- DPN1 digest and gel purify (750 bp product)

- [ ]
    - Miniprep pZA21 from 5 mL YT upstairs

- [x]
    - Do restriction digest with AatII and BamHI in Cutsmart buffer

- [ ]
    - Piece the two together using ligation reaction

- [ ]
    - Transform into Mach 1, and any successful T7pol_pZE21 tacr system.

- [x]
- Make frozen stock of 96well plate with t7RNAPol:pze21 using Jaymin's protocol

- [x]
- Make 52 well EtBR gel for screening T7RNAPol plate.
    - Screen row C using primers 810 and 811. Run for 2:00 minutes at 58 degree annealing time!

- [ ]
- Transform t7-pze21 in mach1 #3 and plate

- [x]
- Transform t7-sfgfp in mach1 and plate

- [x]
- Make 96 well plate of Interlabs and begin screening.

- [ ]
- Make overnight cultures before leaving

- [x]

Friday, July 25, 2014

- Check plates from all the transformations yesterday

- [x]
- Get Strep tag from Rinehart Lab

- [x]
- Order Flag tag from Keck?

- [ ] Get recoded strain for DOPA OTS
- [ ] Mini-Prep cultures grown overnight.
- [ ] Make frozen stock of 96well plate with t7RNAPol:pze21 using Jaymin's protocol
- [x] Make 52 well EtBR gel for screening T7RNAPol plate.
- [ ] Screen row B using primers 810 and 811. Run for 2 minutes at 58 degree annealing time!
- [ ] IPTG into BL21-DE3 that has T7-sfGFP and see if fluorescence occurs
- [x] May not have time to grow up.
- [x] Miniprep of pZA21
- [x] Re-streak Mach1, BL21, BL21-DE3, 576, and DHSalpha
- [x] pZA+T7sfGFP screening with Jaymin's primers: 4 from each strain (Mach1, BL21, BL21 DE3), plus one negative control from each strain (untransformed), plus original plasmid (positive control)
  - [x] PCR amplify at 55˚C
    - [x] Run gel (5 µL)
      - [x] PCR purify and send in for sequencing (Too late today.)
      - [x] Ordered new primer because Jaymin's was pretty close to the T7 promoter...
- [x] Interlab screening: 3 colonies each, plus original pSB1C3 plasmid (from mini prep) and untransformed DH5 alpha.
  - [x] PCR amplify at 55˚C
    - [x] Run gel (5 µL)
      - [ ] PCR purify and send in for sequencing (Too late today.)
- [x] Run restriction digest of T7RNA pol+pZE21: HindIII and KPN1

Sunday, July 27, 2014

- Start Cultures of ECNR2 (LB min), 2 Cultures PZA21 in Mach1(YT), Clones 1-5 (YT), Mach1 control. Let Grow Overnight.
- fron frozen stock row c 1-5 into eppendorf tubes with LB, spec, and iptg.

Monday, July 28, 2014

- Check IPTG assay.
- [ ] In shaking incubator upstairs.
  - As of this morning, not grown yet.
  - Need to have a control
  - Take out 100 uL and put in blue light
  - Grow in canonical tubes
- Miniprep pZA plasmids—all of the culture from BOTH tubes into a single eppendorf, elute with 30 µL.
- [x] Miniprep clones 1-5
- [x] Something doesn't feel right...
- Redo PCR sequencing amplification of clones 1-5 using Ryan's sequencing primers
- [ ] Run 5 µL on a gel
- [ ] Prepare the rest for sequencing

- [ ]
  - Prepare sequencing reactions for Interlab.
- [ ]
  - Start a PCR reaction in HIFI for T7SFGFP with primers 231 and 232. (In case that we don't have enough)
- [x]
    - DPN1 Digest
- [x]
    - Gel purify
- [ ]
    - Nanodrop
- [ ]
    - Digest
- [ ]
    - PCR Purify
- [ ]
    - Ligate
- [ ]
  - Find protocol for integration into genome. Prepare to integrate T7-RR into genome of strain 730(need to get from Natalie)
- [ ]
  - Ask anyone in lab on genome integration
- [x]
      - Jaymin will write up a protocol for us.
  - Run restriction digest with specific enzymes
- [x]
    - PCR purify
- [x]
    - Not enough in both cases, started
    - Nanodrop
- [ ]
    - Ligation
- [ ]
  - T7 RNA Pol for sequencing
- [ ]
    - Gel yielded NOTHING
      - restart cultures for mini prep tomorrow.
- [x]
      - K2G Fast colony PCR with pZE sequencing primers.
- [x]
        - DPN1-digested, left overnight.
  - Make overnight cultures before leaving tonight
- [x]
  - Check the IPTG induced cells in upstairs incubator
- [x]
    - We grew up them wrong. Regrowing them, check tomorrow.

July 29, 2014

- Get pZE21 tacr+T7 RNA pol from PCR 6b and run a gel with 5 µL, and PCR purify the rest.
- [x]
    - Set up sequencing reactions if successful.
- [x]

- Check IPTG assay. Mira will be using the plate reader at 11am.

- [x]

- Miniprep the plasmids—T7 RR (C1-C4), pZA21

- [x]

- PCR purify T7sfGFP (in fridge) and prep for sequencing. Wow, lots of sequencing.

- [x]

- Depending on how the IPTG assay goes, grow up successful BL21 DE3+pZA21_T7sfGFP in 5 mL YT to mini prep tomorrow.

- [x]

- Ask Mira on how to measure fluorescence

- [x]

- Show genotype of Stain 730 to Dr. I

- [ ]

- Order crystal violet staining, establish an assay that works

- [ ]

- Find out more on McFarland standards

- [x]

    - just a scale used for measuring
- Get more information on Strain 730

- [ ]

- Figure out a protocol for the expression assay of the T7-RR expression system so that we can do it as soon as we have both parts ready. (Ideally sometime this week).
    - Read original paper that Dr.Isaacs wrote
    - Talk to Jaymin or Ryan
    - Read Nature paper- 2009 Recombineeering Don Courtz
- See how Ryan uses the fax machine

- [x]

- Bug Jaymin for the sequences to the primers that we used for T7 riboregulation plasmid.

- [x]

- At end of day, prepare overnight cultures

- [x]

- Miniprep successful IPTG assay culture

- [x]

July 30, 2014

- Check transformations.

- [x]

    - pZE21_A12C_T7RNApol (4 plasmids) in ECNR2
    - pZE21_A12C_T7RNApol and messy pZA21_T7sfGFP (4 plasmids vs. 1) in ECNR2
    - clean pZA21_T7RNApol in Mach 1.
- Make frozen stocks of pZE21_A12C_T7RNApol and clean pZA21.

- [x]

- Add all the necessary inducers to the pZE-pZA system and let grow.

- [ ]

- IPTG Assay- Round 1

- [ ]

- At end of day, prepare overnight cultures

- [x]

- Grow 4 pZA21+T7sfGFP clean in Mach1 up in 5 mL of culture to mini prep tomorrow.

July 31, 2014

- IPTG Assay - Round 2

- [ ]

- Miniprep 4 pZA21+T7sfGFP clean in Mach1

- [x]
  - Screen clean pZA21+T7sfGFP
    - PCR using pZA sequencing primers in K2G Fast
- [x]
  - PCR purify
- [x]
  - Send off for sequencing.
- [ ]
  - Will probably need to interpret sequences.
- [ ]
  - Sequences don't look so great. So the riboregulation system needs to be repaired on the T7-RR. We are going to do this by taking an aliquot of the pZE:CAT, digesting out the riboregulation system, doing a partial digest of the T7RNAPol riboregulation system, and inserting the undamaged riboregulation system into the plasmid. We'll see how this goes.
- [ ]
  - Run gel of pZA_CAT
- [x]
  - Gel Purify
- [x]
  - At end of day, prepare overnight cultures
- [x]
  - ECNR2 derive with TolC at 9-10B 13B 21B from Ryan or Jaymin
    - Finish cloning with system without.
  - Design Primers for RK2
- [x] Ariel did this and has them on his computer.
  - At end of day, prepare overnight cultures
- [x]

August 1, 2014

- Prep pZE21_A12C CAT for sequencing
  - Run PCR with Ryan's sequencing primers
  - PCR purify
  - Run gel to verify length
- Run gel for KPN1+Xba1 digest in fridge
  - cut out 300 bp band
  - gel purify
- Miniprep pZE21_A12C cultures (CAT and T7RR 1-3)
- Redo Kpn1+Xba1 digest for pZE21_A12C_T7RR (and possibly pZE CAT)
  - try for 30 minutes at 1/8 and 1/16 dilution for Kpn1 for T7RR
  - Run gel and cut out 5 kb band.
- Learn recombineering from Natalie, put tolC somewhere into 730's genomic hotspots
- [x]
  - Get recoded RK24 from Natalie
- [x]
  - IN ACTUALITY: we transformed into the wrong pZE21 CAT. Which means we have T7RNApol in just regular pZE21
  - Fixing it:
    - Plan A: start from scratch. Reamplify up T7RNApol and pZE21_A12C backbone (correct this time)
      - PCR amplification
- [x]
    - Gel purification
- [x]
      - only for T7pol
    - Digest pZE21_A12C with KPN1 and BamHI
- [ ]
      - Gel purify again
- [ ]
    - Nanodrop

- [ ]
    - Gibson Assemble (3:1 insert:backbone ratio)
- [ ]
    - Drop dialyze
- [ ]
    - Transform into ECNR2

- [ ] alone and with pZA21_T7sfGFP

- Plan B: use restriction enzymes to clone the tacr system inside.
    - mini prep enough pZE21_T7RNApol for restriction digest

- [ ]
    - Amplify tacr system within correct pZE21_A12C_CAT
    - Restriction digest with AatII and KPNI

- [ ]
        - Full one hour for tacr system
        - Partial digest for T7RNApol: do 1/8, 1/16, and, if possible, 1/32 dilutions and run for 15 minutes.
    - Gel purify—tacr system should be 460 bp, and the T7 RNA should be 4.5 kb.
    - Piece the two together via ligation reaction.
    - Drop dialyze
    - Transform into ECNR2

August 2, 2014

- Check incubator for transformations: pZE21_A12C into Mach 1, and pZA+T7sfGFP clean and messy versions into ECNR2. Start cultures to prepare into frozen stock. Looks like a12c failed to transform.

- [x]
- Nanodrop T7pol (labelled w/ green tape)

- [x]
- Check PCR 6a for 6 tubes (various methods of amplifying up the pZE21_A12C backbone with DMSO).
    - Gel purify

- [x]
    - KpnI/BamHI digest #5 and 6

- [x]
        - Pcr purify

- [x]
    - Gibson assemble with T7pol

- [x]
    - Drop dialyze

- [x]
    - If time permitting, transform into ECNR2

- [x]
- Make 2 bottles of agar

- [x]
- Take out the trash

- [x]

August 4, 2014

- [ ] Get pZE21TetG from Ryan
    - pZE21 w/ Tet repressor. Can then use A12C in other strains that do not have both repressors.

- [x] Send a follow-up email to Genscript
- [x] See if we can get a sample of Cell-Tak
- [x] Amplify tolC to put into 13B of genome
- [x] Dpn1 digest
- [x] Gel purify
- [x] pLtetO screening of ECNR2+pZE21_a12C_T7RR
- [x] Run gel
- [x] Select good results and grow up in LBmin with Kan

- Tomorrow: transform pZA21+T7sfGFP into cultures.
- Make overnight cultures

☐
August 5, 2014
☑ Miniprep 4 mL of pZE21_A12C_T7RNApol cultures.
☑ Run restriction digest assay
☑ Run result on gel

- Success!

☑ Transform pZA21_T7sfGFP (messy because clean version has not been screened) into four pZE21_A12C_T7RNApol strains.
☑ Check transformations—pZE21_a12C_CAT and pZE21_tolC
☑ start liquid cultures
☑ Take PCR purified product and prep for sequencing (make sure to place orders on Keck)
☑ Learn how to wash the dishes
☑ Recombineer tolC into strain #730
☐ let recover for 2 hours and then plate on SDS
☐ RK24 Assembly- see separate note- Yamini is working on

August 6, 2014

- Transformations failed in two different ways—pZE21_A12C_T7RNApol + pZA21_T7sfGFP overgrew, streaked out plates to check on tomorrow, and retransforming one available culture (B2, made into frozen stock)
- tolC genome recombineering did NOT grow in 3/4 strains: redoing #730, 731, and 331.
  - 331 was the only one that grew up to mid log, so that one was recombineered. 730 and 731 are going to grow overnight and get transferred to new cultures tomorrow.
- Primers for amplification of T7 RR with 13B genomic overhangs ordered.
- Will screen for tolC in 120 and 331 (hopefully) tomorrow

August 7, 2014
☑ Start growing cultures for GFP assay: start up B2 A12C_T7RNApol+pZA21_T7sfGFP in Kan+Spe, B2 A12C_T7RNApol in Kan, pZA21_T7sfGFP in Spe, and ECNR2 in plain. All plasmids should be in ECNR2 strain.
☐ After they've grown for two hours or so, transfer them to plate with fluid conditions ready. That's IPTG+ATC, IPTG only, ATC only, and nothing.
☑ Run sequencing reactions for Interlab construct #3 again.
☑ Run gel
☐ PCR purify products
☑ Recombineer strains 730 and 731.

- Currently recovering on bench top. Plate tomorrow.

August 8, 2014

- Plate 730 and 731 recombineered cultures. ☑
- A12C_T7RNApol recombineering fragment generation:
  - Currently in PCR6a. First, DpnI digest ☑
- Gel purify ☑
  - Ran for too long but still got a band.
- Nanodrop ☐
- Also nanodrop pZE21_A12C_T7RNApol miniprep. Pretty sure there's stuff there but not positive.
- GFP fluorescence assay ☑
  - Will redo on Monday

August 11, 2014
☐ Order column and Strep tag
☑ Redo GFP Assay
☑ Grow up A12C+pZA, A12C, pZA, and ECNR2 in 96-well plate w/ correct antibiotics.
☑ Set up inducers in separate wells.
☑ When original ones are cloudy, transfer to screening plate.
☑ Screen #730 and #731 for tolC

- 96 well plate set up, PCR going happily

☐ Attempt to recombineer in the afternoon if successful.
☑ Transfer 100 µL of culture from successfully screened colony into a culture flask and let grow until mid log. Transform with T7RR fragment created on Friday.

August 12, 2014

- Remake A12C+pZA from scratch and by transforming A12C into pZA.
  - Miniprep out pZE21/pZA21 T7sfGFP. The pZE21 version is for eventual
  - Transform ECNR2 with both plasmids, and transform ECNR2+pZA21_T7sfGFP with A12C_T7RR.
    - Low 4.4-4.9 time constants.
- ~~Set up a 96-well plate with previous pZA/A12C transformation and pray one works.~~
- Do negative selection with recombineered 730/731. Both plate and liquid version.
  - 731 took some time to grow, we'll see how it fares.
  - Plates are finished (the volume of colison used is suspect...), will do liquid selection tomorrow.

August 13, 2014

- Check A12C-pZA transformations.
  - haha sucker nothing worked. REDO REDO
    - retransformed into A12C with both pZA and pZE plasmid variations just in case I messed up the labeling last time—time constants 4.8, 5.0 and 5.0 (control). Plated on Kan+Spe
- PCR Purify E5 and E6 of interlab and send them in for sequencing.
  - Also didn't work. Growing up in culture to do tomorrow.
- Genome integration negative selection assay: 2 plated on Spe, 2 still going.
- Make SDS and Cat Plates

August 14, 2014

- Action Items based on Lab Meeting:
  - challenge plates with pZA
  - take an ancestral plasmid
  - battery of controls
  - optimizing concentration of antibiotics
    - test 15-30%
    - decreasing copy # of spec
    - batch of plates with different concentration
- Advice for Low Yield of T7 RR with TolC:
  - elute in 100
  - pre-heat water
- PCR Interlab 3 from culture media and send off for sequencing: E3, E4, and E5 to Genewhiz.
- Recombineered 730 and 731 again.
- Retransformed pZA-A12C system again, and plated on new plates.

August 15, 2014

- Check A12C-pZA transformations. Do PCR screening because there isn't any time to set up the GFP assay (research weekend lines maybe). Haha never mind, the lesson here is never plate while drunk.
  - Screen the plasmids. 810/811 for A12C (size should be the indicator) and 13B for the single 731 colony that grew on the experimental plate. Also screening pZE/pZE T7sfGFP for Spec resistance.
    - 100% certain on A12C plasmid, about 75% sure on pZA plasmid.
  - Something grew on the pZA control plates. Starting a culture of one of the colonies with both resistance markers, and including them on above screenings. Using previous pZE/pZA ECNR2 strain (with incorrect pZE plasmid) as positive control.
  -
- Select for tolC knockout in both the kinetics assay and plating 50 µL on ColE1 plates.
  - Plated kinetics assay on plain LB plates. Cultures were not very cloudy, so did 10^-3 for 1.5 inoculation and 10^-4 for 15 inoculation.
- Interlab sequencing returned, I have to do this again...

August 16, 2014

- Redoing Interlab 3:
  - Miniprep out Plasmid 2 ☑
  -
  - PCR using primers 198/200 ☑
  - **DPN1 Digest** ☑
  - Gel purify ☑
    - Weird band at 1 kb, has seen this before.
  - Drop Dialyze ☑
  - Nanodrop ☑
    - yield of 16.8 ng/uL.
- Transform ECNR2+pZA and Mach1+pZA with A12C ☑
    - Also transformed strains #568 and #120 (NJM database) with both plasmids.
  - Let recover for at least 1:20, and plate on Kan+Spe ☑
    - used fresh plates made with fresh antibiotics.
- Screen genome/pRK24 integration, check plates first ☑
  - Plates success, screening less so. 96 well plate started.

August 17, 2014

- Check T7 ~~and interlab~~ transformations ☑
  - Start cultures for GFP assay ☑
- Interlab 3:
  - Start up a culture of Mach1 ☑
  - Transform into Mach1 and plate on CAT plates ☑
- Screening T7 genome: use fresh stocks of internal primer and one of the T7 generating primers (195 or 196). Use A12C as a positive control.
  - Make a 4-row EtBr gel to screen.
  - All negative, but positive control works.

August 18, 2014

- Natalie will potentially redo T7 characterization TF
- Amplify Interlab 3 for sequencing ☐
- Set up GFP assay ☐
- Pick new colonies to screen for T7

August 19, 2014

- Conduct GFP assay☐
- Get Interlab 3 sequencing data, start culture and transform correct plasmid into DH5A☐
  - Also transform premade construct (Interlab 1) into DH5A☐
- Transform the DOPA OTS into SOMETHING.☐
- Nothing of the sort happened. Sent in Interlab 3 for sequencing. Screened double plasmid construct and found positive results.

August 22, 2014

- Set up GFP assay cultures.
- Started cultures of Interlab 3 A3 and chloramphenicol control, ECNR2+pZE21_A12C_T7RNApol and kanamycin control to mini prep plasmids, and ECNR2+pZE21_A12C_T7RNApol+pZA21_T7sfGFP for frozen stock, plus spectinomycin control.
- Ran PCR of the T7 genomic integration—in fridge

August 23, 2014

- Miniprep A12C and Interlab plasmids☑
  - Make frozen stock of the Interlab and the A12C+pZA strain☑
- Transform DH5A with Interlab plasmid☑
  - Make chloramphenicol plates before transformation☐
    - Don't put the chloramphenicol plates in the freezer.
- DPN1 digest genomic T7RR☑
  - Run on gel and ~~gel purify~~☑
- Set up GFP fluorescence assay☑

August 24, 2014

- Plate Interlab 3 transformation.☐
  - Need to make Cam plates first.☑
  - I SHOULD HAVE ALSO TRANSFORMED CONSTRUCT #1 I'M A DINGUS
    - Plasmid currently being drop dialyzed downstairs.
- Gel purify T7RR genome amplicon☑
- Screen GFP assay☑

August 25, 2014

- Do the quick GFP assay with only the fully induced construct, the positive and negative controls to see if the system works at all.
- Order sets of sequencing primers to sequence ALL of T7 RNA pol.

August 27, 2014

- Make frozen stock of the successful DH5A+Interlab 3 transformation.
- Transform Interlab 1 into DH5A. Culture tube, DNA/control, and recovery media labeled on styrofoam holder downstairs.
  - Dh5a never became cloudy. Putting some into incubator for overnight growth. Transformation must be done tomorrow

TO DO

Author: stephanie_mao

July 18, 2014

- Autoclave

☑

- Transformation of T7-GFP-pZA15

☑

  - need to wait until agar plates are made with spec
  - Plate it

☑

- Make more culture of Mach1 and interlab alpha culture-yamini

☑

- Start a Yt culture of T7 Promoter

☑

- Mini-prep T7 RNA pol samples (11), Nanodrop plasmid concentrations.

☑

- Run restriction enzyme digest (1 hour protocol, cut buffer) with KPN1 and HindIII and run gel.

☑

- Run gel on massive PCR done with Ryan's sequencing primers/Internal primers combination.
- (Should the primers get here) Get T7+sfGFP into pZA21

☑

- Redo Interlab study

- [ ]
    - Kappa Fast PCR with 197/199 for promoter 3, and 198/200 for promoter 2
- [x]
    - DPN1 digest
- [x]
    - Run gel to test correct fragment length
- [x]
    - Redo 3
    - Gibson assemble.
- [ ]
    - Drop dialyze
- [ ]
    - Transform into DSH alpha
- [ ]

- Keep on looking up respective areas (Stephanie-constructs, Yamini-Biofilm assays, Ariel-Extraction protocol)
- [x]

- Finalizing Polymer Design- Yamini
- [x]

- Put away the Kan in freezer before leaving the lab for the day
- [x]

July 21, 2014

- Kappa Fast PCR with 219/220 for T7+sfGFP, and 221/222 for pZA21 (mini prep plasmid)
- [ ]
        - 220 wrong, forgot that I'm keeping in a bit of the old GFP, reordered primer.
    - DPN1 digest
- [ ]
    - Gel purify
- [ ]
    - Gibson assemble
- [ ]
    - Drop dialyze
- [ ]
    - Transform into Mach 1
- [ ]

- Miniprep culture that I started but completely forgot what the heck it was, sorry (Stephanie) (On bench)
- [x]

- Check pZA21 plates on bench.
- [ ]

- Redo Interlab study
- [x]
    - May have to re-transform plasmid if I don't have a strain with just that plasmid, although I recall someone doing that transformation earlier. Maybe it failed...
        - Update: has strain, doing PCR from colony and starting culture for miniprep
    - Kappa HiFi Two-step PCR with 197/199 for promoter 3, and 198/200 for promoter 2 (use cell from frozen stock)
- [x]
    - DPN1 digest
- [x]

- Run gel to test correct fragment length

- [x]
  - Redo 3
  - Gibson assemble.
- [x]
  - Drop dialyze
- [x]
  - Transform into DSH alpha
- [ ]

- Edit constructs to get rid of repeating sequence
- [x]
- Spec plates for pZA transformation, more agar just in case (we may need Kan+CAT, or Kan+IPTG, etc.
- [ ]
- Design primers to extract constructs when they get here.
- [x]
- Get DOPA OTS construct from Mira
- [x]
  - or we might have to make tyrosine OTS from the DOPA OTS
- [ ]
- Send original primer designs and sequences to Dr. Isaacs
- [ ]
    - Ariel planning to meet with Dr. Isaacs at 3:15
- Get oriented directly with Genscript contact, no middlemen
- [x]
- Cut plasmid with just XHO1 and Kpn1, or do a double digest to see if we get 2 bands
- [ ]
- Ask Jaymin about sequences we got from and then do a digest
- [ ]
- Look at DNA 2.0: find a good algorithm to optimize expression. Ask them if they can run it through their optimization system
- [x]
    - tell them we are iGEM students
- Ask Lexi for strain for tyrosine amber suppressor
- [ ]
    - or we can mutate with Mage oligos
    - use Mage to convert to Tyr Amber suppresor
- Check that P. Arginosa is BL1
- [x]
    - Yes it is BL1
- Look at more literature on biofilm assays
- [x]
    - Couldn't find any paper that uses MAPs and the MBEC assay
- More research on purification with tags
    - possible options:
    - Flag tag with MBP
- [ ]
  - Talk to Mira about ELP (using a salt gradient)
- [ ]
  - Is GST only on N-terminus?
- [x] AND C-Terminus
  - Dual step and flag tag - do a lit. search

- [x] This has happened.
    - see how people have used tags in combination, and look into whether N or C terminus specifics
- [ ]
- Set up with mtg with Dr. Isaacs tomorrow or Wed. to go into more detail on protein purification
- [ ]
    - Need to have a rough idea of top 2 tags
- T7_GFP:
- [ ]
    - Do with restriction enzymes
    - clean gene replacement
    - linear region just 16-18 base pairs
    - Try- our method, but also with restriction enzymes and quick-ligase protocol
- Look at NEB:
- [ ]
    - What bands look like on gel
    - resolving band lengths
- Get a recoded strain for rE.Coli OTS
- [ ]
- DOPA OTS system Miniprep out when we get it from Mira
- [ ]
- Make CAM Plates
- [ ]
- Look at Mira's ELP paper
- [ ]

July 22, 2014

- Conduct KPN1 digest of plasmid. Protocol included below. Continue cloning protocol for T7RR
- [ ]
    - Reaction Mixture
        - 35uL DNA from tube labeled for KPN1
        - 1 uL Enzyme
        - 4 uL Buffer
        - See Jaymin's protocol on sticky note in office to verify.
    - PCR purify post Digest. Now ready to Gibson Assemble.
- Fill water bath with water. Do not adjust temperature
- [ ]
- Order primers for restriction assembly of pZA21+T7sfGFP
- [x]
- Redo interlab 3 amplification and purification
- [x]
- Begin T7 promoter:sfgfp- pZA21 assembly with primers.
- [x]
- Begin new T7RR assembly protocol with primer 196.
- [x]
- Develop screening protocols for tomorrow. Will have a lot to screen.
- [ ]
- Make some fresh CAT
- [x]
- Prepare DOPA OTS and tyrosine supressor system
- [x]
- Look at NEB:
- [x]
    - What bands look like on gel
    - resolving band lengths

- T7_GFP:

- [ ]
    - Do with restriction enzymes
    - clean gene replacement
    - linear region just 16-18 base pairs
    - Try- our method, but also with restriction enzymes and quick-ligase protocol
- Set up with mtg with Dr. Isaacs tomorrow or Wed. to go into more detail on protein purification

- [x]
    - Need to have a rough idea of top 2 tags
    - Also go over our top 3 track selection
- More research on purification with tags- Yamini is working on
    - possible options:
    - Flag tag with MBP

- [x]
    - Talk to Mira about ELP (using a salt gradient)

- [x]
    - Is GST only on N-terminus?

- [x] AND C-Terminus
    - Dual strep and flag tag - do a lit. search

- [x] This has happened.
    - see how people have used tags in combination, and look into whether N or C terminus specifics

- [x]
- Ask Jaymin about sequences we got from and then do a digest

- [x]
- Ariel: On the gel you're running, the T7sfGFP will be 786 bp, and the pZE tacr backbone will be 2600 bp.

July 23, 2014

- Check T7_pZE21 Plates Round 1 and 2 tomorrow

- [x]
- transformation of overnight drop dialysis of T7_pZE21 into Mach 1

- [ ]
    - plate it on Kan plates

- [ ]
- Check overnight PCR of pZE21 tacr backbone in PCR 6

- [x]
    - DPN1 digest

- [ ]
    - Gel purify

- [ ] (fragment length of 2600 bp)
    - Nanodrop

- [ ]
    - Gibson assemble with T7 fragment

- [ ]
    - Transform into Mach 1 strain

- [ ]
    - Plate on Kan

- [ ]
- Gibson together T7sfGFP and pZA21

- [x]
    - Transform into Mach 1 and successful T7pol+pZE21 if available.

- [ ]

- Transform Interlab into DS5 alpha

- [ ]

- Primers for the clean pZA21+T7sfGFP should arrive:
  - PCR amplify (225/226), too short for two-step

- [x]

  - Gel purify

- [ ]

- Not enough pZA21 for restriction digest—inoculated 5 mL of YT with Mach 1 + pZA21
  - Restriction digest with AatII and BamHI+ligation

- [ ]

  - Drop dialyze

- [ ]

  - Transform into successful T7pol+pZE21 and Mach 1

- [ ]

- Email Karl and Natash on SF-TAP protocol

- [x]

- Get P.Arginosa from Turner lab

- [ ]

- Put Gibson Ass. Mix cells in -80 degrees

- [x]

- Send Genotype information to Dr. Isaacs

- [x]

  - Make a streak so we have a frozen stock
- Protocol for LL-37 to Titanium

- [x]

July 24, 2014

- Constructing clean t7Promsfgfp_pZA21

- [ ]

  - Redo T7sfGFP PCR using HiFi, primers 225 and 226, with annealing temp at 54˚C and 45s extension time.

(Primers and template on Stephs bench)

  - DPN1 digest and gel purify (750 bp product)

- [ ]

  - Miniprep pZA21 from 5 mL YT upstairs

- [x]

  - Do restriction digest with AatII and BamHI in Cutsmart buffer

- [ ]

  - Piece the two together using ligation reaction

- [ ]

  - Transform into Mach 1, and any successful T7pol_pZE21 tacr system.

- [x]

- Make frozen stock of 96well plate with t7RNAPol:pze21 using Jaymin's protocol

- [x]

- Make 52 well EtBR gel for screening T7RNAPol plate.
  - Screen row C using primers 810 and 811. Run for 2:00 minutes at 58 degree annealing time!

- [ ]

- Transform t7-pze21 in mach1 #3 and plate

- [x]

- Transform t7-sfgfp in mach1 and plate

- [x]

- Make 96 well plate of Interlabs and begin screening.

- [ ]

- Make overnight cultures before leaving

- [x]

Friday, July 25, 2014

- Check plates from all the transformations yesterday

- [x]

- Get Strep tag from Rinehart Lab

- [x]

- Order Flag tag from Keck?

- [ ]

- Get recoded strain for DOPA OTS

- [ ]

- Mini-Prep cultures grown overnight.

- [ ]

- Make frozen stock of 96well plate with t7RNAPol:pze21 using Jaymin's protocol

- [x]

- Make 52 well EtBR gel for screening T7RNAPol plate.

- [ ]

    - Screen row B using primers 810 and 811. Run for 2 minutes at 58 degree annealing time!
- IPTG into BL21-DE3 that has T7-sfGFP and see if fluorescence occurs

- [x]

    - May not have time to grow up.
- Miniprep of pZA21

- [x]

- Re-streak Mach1, BL21, BL21-DE3, 576, and DHSalpha

- [x]

- pZA+T7sfGFP screening with Jaymin's primers: 4 from each strain (Mach1, BL21, BL21 DE3), plus one negative control from each strain (untransformed), plus original plasmid (positive control)
    - PCR amplify at 55˚C

- [x]

    - Run gel (5 µL)

- [x]

    - PCR purify and send in for sequencing (Too late today.)
    - Ordered new primer because Jaymin's was pretty close to the T7 promoter...
- Interlab screening: 3 colonies each, plus original pSB1C3 plasmid (from mini prep) and untransformed DH5 alpha.
    - PCR amplify at 55˚C

- [x]

    - Run gel (5 µL)

- [ ]

    - PCR purify and send in for sequencing (Too late today.)
- Run restriction digest of T7RNA pol+pZE21: HindIII and KPN1

- [x]

Sunday, July 27, 2014

- Start Cultures of ECNR2 (LB min), 2 Cultures PZA21 in Mach1(YT), Clones 1-5 (YT), Mach1 control. Let Grow Overnight.
- fron frozen stock row c 1-5 into eppendorf tubes with LB, spec, and iptg.

Monday, July 28, 2014

- Check IPTG assay.

- [ ] In shaking incubator upstairs.

    - As of this morning, not grown yet.
    - Need to have a control
    - Take out 100 uL and put in blue light

    - Grow in canonical tubes
- Miniprep pZA plasmids—all of the culture from BOTH tubes into a single eppendorf, elute with 30 µL.

- [x]

- Miniprep clones 1-5

- [x]

    - Something doesn't feel right...
- Redo PCR sequencing amplification of clones 1-5 using Ryan's sequencing primers

- [ ]

    - Run 5 µL on a gel

- [ ]

    - Prepare the rest for sequencing

- [ ]

- Prepare sequencing reactions for Interlab.

- [ ]

- Start a PCR reaction in HIFI for T7SFGFP with primers 231 and 232. (In case that we don't have enough)

- [x]

    - DPN1 Digest

- [x]

    - Gel purify

- [ ]

    - Nanodrop

- [ ]

    - Digest

- [ ]

    - PCR Purify

- [ ]

    - Ligate

- [ ]

- Find protocol for integration into genome. Prepare to integrate T7-RR into genome of strain 730(need to get from Natalie)

- [ ]

- Ask anyone in lab on genome integration

- [x]

    - Jaymin will write up a protocol for us.
- Run restriction digest with specific enzymes

- [x]

    - PCR purify

- [x]

    - Not enough in both cases, started
    - Nanodrop

- [ ]

    - Ligation

- [ ]

- T7 RNA Pol for sequencing

- [ ]

    - Gel yielded NOTHING
        - restart cultures for mini prep tomorrow.

- [x]

        - K2G Fast colony PCR with pZE sequencing primers.

- [x]

        - DPN1-digested, left overnight.
- Make overnight cultures before leaving tonight

- [x]

- Check the IPTG induced cells in upstairs incubator

- [x]

    - We grew up them wrong. Regrowing them, check tomorrow.

July 29, 2014

- Get pZE21 tacr+T7 RNA pol from PCR 6b and run a gel with 5 µL, and PCR purify the rest.

- [x]

    - Set up sequencing reactions if successful.

- [x]

- Check IPTG assay. Mira will be using the plate reader at 11am.

- [x]

- Miniprep the plasmids—T7 RR (C1-C4), pZA21

- [x]

- PCR purify T7sfGFP (in fridge) and prep for sequencing. Wow, lots of sequencing.

- [x]

- Depending on how the IPTG assay goes, grow up successful BL21 DE3+pZA21_T7sfGFP in 5 mL YT to mini prep tomorrow.

- [x]

- Ask Mira on how to measure fluorescence

- [x]

- Show genotype of Stain 730 to Dr. I

- [ ]

- Order crystal violet staining, establish an assay that works

- [ ]

- Find out more on McFarland standards

- [x]

    - just a scale used for measuring
- Get more information on Strain 730

- [ ]

- Figure out a protocol for the expression assay of the T7-RR expression system so that we can do it as soon as we have both parts ready. (Ideally sometime this week).
    - Read original paper that Dr.Isaacs wrote
    - Talk to Jaymin or Ryan
    - Read Nature paper- 2009 Recombineeering Don Courtz
- See how Ryan uses the fax machine

- [x]

- Bug Jaymin for the sequences to the primers that we used for T7 riboregulation plasmid.

- [x]

- At end of day, prepare overnight cultures

- [x]

- Miniprep successful IPTG assay culture

- [x]

July 30, 2014

- Check transformations.

- [x]

    - pZE21_A12C_T7RNApol (4 plasmids) in ECNR2
    - pZE21_A12C_T7RNApol and messy pZA21_T7sfGFP (4 plasmids vs. 1) in ECNR2
    - clean pZA21_T7RNApol in Mach 1.
- Make frozen stocks of pZE21_A12C_T7RNApol and clean pZA21.

- [x]

- Add all the necessary inducers to the pZE-pZA system and let grow.

- [ ] IPTG Assay- Round 1
- [ ] At end of day, prepare overnight cultures
- [x] Grow 4 pZA21+T7sfGFP clean in Mach1 up in 5 mL of culture to mini prep tomorrow.

July 31, 2014

- [ ] IPTG Assay - Round 2
- [ ] Miniprep 4 pZA21+T7sfGFP clean in Mach1
- [x] Screen clean pZA21+T7sfGFP
  - PCR using pZA sequencing primers in K2G Fast
- [x] PCR purify
- [x] Send off for sequencing.
- [ ] Will probably need to interpret sequences.
- [ ] Sequences don't look so great. So the riboregulation system needs to be repaired on the T7-RR. We are going to do this by taking an aliquot of the pZE:CAT, digesting out the riboregulation system, doing a partial digest of the T7RNAPol riboregulation system, and inserting the undamaged riboregulation system into the plasmid. We'll see how this goes.
- [ ] Run gel of pZA_CAT
- [x] Gel Purify
- [x] At end of day, prepare overnight cultures
- [x] ECNR2 derive with TolC at 9-10B 13B 21B from Ryan or Jaymin
  - Finish cloning with system without.
- Design Primers for RK2

[x] Ariel did this and has them on his computer.

- At end of day, prepare overnight cultures

[x]

August 1, 2014

- Prep pZE21_A12C CAT for sequencing
  - Run PCR with Ryan's sequencing primers
  - PCR purify
  - Run gel to verify length
- Run gel for KPN1+Xba1 digest in fridge
  - cut out 300 bp band
  - gel purify
- Miniprep pZE21_A12C cultures (CAT and T7RR 1-3)
- Redo Kpn1+Xba1 digest for pZE21_A12C_T7RR (and possibly pZE CAT)
  - try for 30 minutes at 1/8 and 1/16 dilution for Kpn1 for T7RR
  - Run gel and cut out 5 kb band.
- Learn recombineering from Natalie, put tolC somewhere into 730's genomic hotspots
- [x] Get recoded RK24 from Natalie
- [x] IN ACTUALITY: we transformed into the wrong pZE21 CAT. Which means we have T7RNApol in just regular pZE21
- Fixing it:

- Plan A: start from scratch. Reamplify up T7RNApol and pZE21_A12C backbone (correct this time)
  - PCR amplification

- [x]
  - Gel purification
- [x]
    - only for T7pol
  - Digest pZE21_A12C with KPN1 and BamHI
- [ ]
    - Gel purify again
- [ ]
  - Nanodrop
- [ ]
  - Gibson Assemble (3:1 insert:backbone ratio)
- [ ]
  - Drop dialyze
- [ ]
  - Transform into ECNR2

- [ ] alone and with pZA21_T7sfGFP

- Plan B: use restriction enzymes to clone the tacr system inside.
  - mini prep enough pZE21_T7RNApol for restriction digest

- [ ]
  - Amplify tacr system within correct pZE21_A12C_CAT
  - Restriction digest with AatII and KPNI
- [ ]
    - Full one hour for tacr system
    - Partial digest for T7RNApol: do 1/8, 1/16, and, if possible, 1/32 dilutions and run for 15 minutes.
  - Gel purify—tacr system should be 460 bp, and the T7 RNA should be 4.5 kb.
  - Piece the two together via ligation reaction.
  - Drop dialyze
  - Transform into ECNR2

August 2, 2014

- Check incubator for transformations: pZE21_A12C into Mach 1, and pZA+T7sfGFP clean and messy versions into ECNR2. Start cultures to prepare into frozen stock. Looks like a12c failed to transform.

- [x]
- Nanodrop T7pol (labelled w/ green tape)

- [x]
- Check PCR 6a for 6 tubes (various methods of amplifying up the pZE21_A12C backbone with DMSO).
  - Gel purify

- [x]
  - KpnI/BamHI digest #5 and 6
- [x]
    - Pcr purify

- [x]
  - Gibson assemble with T7pol

- [x]
  - Drop dialyze

- [x]
  - If time permitting, transform into ECNR2

- [x]
- Make 2 bottles of agar

- [x]

- Take out the trash

☑

August 4, 2014
☐ Get pZE21TetG from Ryan

- pZE21 w/ Tet repressor. Can then use A12C in other strains that do not have both repressors.

☑ Send a follow-up email to Genscript
☑ See if we can get a sample of Cell-Tak
☑ Amplify tolC to put into 13B of genome
☑ Dpn1 digest
☑ Gel purify
☑ pLtetO screening of ECNR2+pZE21_a12C_T7RR
☑ Run gel
☑ Select good results and grow up in LBmin with Kan

- Tomorrow: transform pZA21+T7sfGFP into cultures.

- Make overnight cultures

☐
August 5, 2014
☑ Miniprep 4 mL of pZE21_A12C_T7RNApol cultures.
☑ Run restriction digest assay
☑ Run result on gel

- Success!

☑ Transform pZA21_T7sfGFP (messy because clean version has not been screened) into four pZE21_A12C_T7RNApol strains.
☑ Check transformations—pZE21_a12C_CAT and pZE21_tolC
☑ start liquid cultures
☑ Take PCR purified product and prep for sequencing (make sure to place orders on Keck)
☑ Learn how to wash the dishes
☑ Recombineer tolC into strain #730
☐ let recover for 2 hours and then plate on SDS
☐ RK24 Assembly- see separate note- Yamini is working on

August 6, 2014

- Transformations failed in two different ways—pZE21_A12C_T7RNApol + pZA21_T7sfGFP overgrew, streaked out plates to check on tomorrow, and retransforming one available culture (B2, made into frozen stock)
- tolC genome recombineering did NOT grow in 3/4 strains: redoing #730, 731, and 331.
  - 331 was the only one that grew up to mid log, so that one was recombineered. 730 and 731 are going to grow overnight and get transferred to new cultures tomorrow.
- Primers for amplification of T7 RR with 13B genomic overhangs ordered.
- Will screen for tolC in 120 and 331 (hopefully) tomorrow

August 7, 2014
☑ Start growing cultures for GFP assay: start up B2 A12C_T7RNApol+pZA21_T7sfGFP in Kan+Spe, B2 A12C_T7RNApol in Kan, pZA21_T7sfGFP in Spe, and ECNR2 in plain. All plasmids should be in ECNR2 strain.
☐ After they've grown for two hours or so, transfer them to plate with fluid conditions ready. That's IPTG+ATC, IPTG only, ATC only, and nothing.
☑ Run sequencing reactions for Interlab construct #3 again.
☑ Run gel
☐ PCR purify products
☑ Recombineer strains 730 and 731.

- Currently recovering on bench top. Plate tomorrow.

August 8, 2014

- Plate 730 and 731 recombineered cultures. ☑
- A12C_T7RNApol recombineering fragment generation:
  - Currently in PCR6a. First, DpnI digest ☑
- Gel purify ☑
  - Ran for too long but still got a band.
- Nanodrop ☐
- Also nanodrop pZE21_A12C_T7RNApol miniprep. Pretty sure there's stuff there but not positive.
- GFP fluorescence assay ☑
  - Will redo on Monday

August 11, 2014
☐ Order column and Strep tag
☑ Redo GFP Assay
☑ Grow up A12C+pZA, A12C, pZA, and ECNR2 in 96-well plate w/ correct antibiotics.
☑ Set up inducers in separate wells.
☑ When original ones are cloudy, transfer to screening plate.
☑ Screen #730 and #731 for tolC

- 96 well plate set up, PCR going happily

☐ Attempt to recombineer in the afternoon if successful.
☑ Transfer 100 µL of culture from successfully screened colony into a culture flask and let grow until mid log. Transform with T7RR fragment created on Friday.

August 12, 2014

- Remake A12C+pZA from scratch and by transforming A12C into pZA.
  - Miniprep out pZE21/pZA21 T7sfGFP. The pZE21 version is for eventual
  - Transform ECNR2 with both plasmids, and transform ECNR2+pZA21_T7sfGFP with A12C_T7RR.
    - Low 4.4-4.9 time constants.
- ~~Set up a 96-well plate with previous pZA/A12C transformation and pray one works.~~
- Do negative selection with recombineered 730/731. Both plate and liquid version.
  - 731 took some time to grow, we'll see how it fares.
  - Plates are finished (the volume of colison used is suspect…), will do liquid selection tomorrow.

August 13, 2014

- Check A12C-pZA transformations.
  - haha sucker nothing worked. REDO REDO
    - retransformed into A12C with both pZA and pZE plasmid variations just in case I messed up the labeling last time—time constants 4.8, 5.0 and 5.0 (control). Plated on Kan+Spe
- PCR Purify E5 and E6 of interlab and send them in for sequencing.
  - Also didn't work. Growing up in culture to do tomorrow.
- Genome integration negative selection assay: 2 plated on Spe, 2 still going.
- Make SDS and Cat Plates

August 14, 2014

- Action Items based on Lab Meeting:
  - challenge plates with pZA
  - take an ancestral plasmid
  - battery of controls
  - optimizing concentration of antibiotics
    - test 15-30%
    - decreasing copy # of spec
    - batch of plates with different concentration
- Advice for Low Yield of T7 RR with TolC:
  - elute in 100
  - pre-heat water
- PCR Interlab 3 from culture media and send off for sequencing: E3, E4, and E5 to Genewhiz.
- Recombineered 730 and 731 again.
- Retransformed pZA-A12C system again, and plated on new plates.

August 15, 2014

- Check A12C-pZA transformations. Do PCR screening because there isn't any time to set up the GFP assay (research weekend lines maybe). Haha never mind, the lesson here is never plate while drunk.
  - Screen the plasmids. 810/811 for A12C (size should be the indicator) and 13B for the single 731 colony that grew on the experimental plate. Also screening pZE/pZE T7sfGFP for Spec resistance.
    - 100% certain on A12C plasmid, about 75% sure on pZA plasmid.
  - Something grew on the pZA control plates. Starting a culture of one of the colonies with both resistance markers, and including them on above screenings. Using previous pZE/pZA ECNR2 strain (with incorrect pZE plasmid) as positive control.
  -
- Select for tolC knockout in both the kinetics assay and plating 50 µL on ColE1 plates.
  - Plated kinetics assay on plain LB plates. Cultures were not very cloudy, so did 10^-3 for 1.5 inoculation and 10^-4 for 15 inoculation.
- Interlab sequencing returned, I have to do this again…

August 16, 2014

- Redoing Interlab 3:
  - Miniprep out Plasmid 2 ☑
  -
  - PCR using primers 198/200 ☑
  - **DPN1 Digest** ☑
  - Gel purify ☑
    - Weird band at 1 kb, has seen this before.
  - Drop Dialyze ☑
  - Nanodrop ☑
    - yield of 16.8 ng/uL.
- Transform ECNR2+pZA and Mach1+pZA with A12C ☑
    - Also transformed strains #568 and #120 (NJM database) with both plasmids.
  - Let recover for at least 1:20, and plate on Kan+Spe ☑
    - used fresh plates made with fresh antibiotics.
- Screen genome/pRK24 integration, check plates first ☑
  - Plates success, screening less so. 96 well plate started.

August 17, 2014

- Check T7 ~~and interlab~~ transformations ☑
  - Start cultures for GFP assay ☑
- Interlab 3:
  - Start up a culture of Mach1 ☑
  - Transform into Mach1 and plate on CAT plates ☑
- Screening T7 genome: use fresh stocks of internal primer and one of the T7 generating primers (195 or 196). Use A12C as a positive control.
  - Make a 4-row EtBr gel to screen.
  - All negative, but positive control works.

August 18, 2014

- Natalie will potentially redo T7 characterization TF
- Amplify Interlab 3 for sequencing ☐
- Set up GFP assay ☐
- Pick new colonies to screen for T7

August 19, 2014

- Conduct GFP assay ☐
- Get Interlab 3 sequencing data, start culture and transform correct plasmid into DH5A ☐
  - Also transform premade construct (Interlab 1) into DH5A ☐
- Transform the DOPA OTS into SOMETHING. ☐
- Nothing of the sort happened. Sent in Interlab 3 for sequencing. Screened double plasmid construct and found positive results.

August 22, 2014

- Set up GFP assay cultures.
- Started cultures of Interlab 3 A3 and chloramphenicol control, ECNR2+pZE21_A12C_T7RNApol and kanamycin control to mini prep plasmids, and ECNR2+pZE21_A12C_T7RNApol+pZA21_T7sfGFP for frozen stock, plus spectinomycin control.
- Ran PCR of the T7 genomic integration—in fridge

August 23, 2014

- Miniprep A12C and Interlab plasmids ☑
  - Make frozen stock of the Interlab and the A12C+pZA strain ☑
- Transform DH5A with Interlab plasmid ☑
  - Make chloramphenicol plates before transformation ☐
    - Don't put the chloramphenicol plates in the freezer.
- DPN1 digest genomic T7RR ☑
  - Run on gel and ~~gel purify~~ ☑
- Set up GFP fluorescence assay ☑

August 24, 2014

- Plate Interlab 3 transformation. ☑
  - Need to make Cam plates first. ☑
  - I SHOULD HAVE ALSO TRANSFORMED CONSTRUCT #1 I'M A DINGUS
    - Plasmid currently being drop dialyzed downstairs.
- Gel purify T7RR genome amplicon ☑
- Screen GFP assay ☑

RK24 Assembly

Map
Author: Ariel Hernandez-Leyva
Protocol (Creating huge plasmid with a small copy number)

Make a GelGreen gel
Re-run DMSO and 2-Step

- Obtaining Fragments
  - Dilute primers Working stock solutions 10 and 90 ☑
  - Amplify TolC from strain containing RK24:tolc that Natalie provided us with primers A12C_tol.c_R and RK24_tol.c_F ☑
    - PCR Conditions 57 or 58 degrees for 2 minutes (1,650 bp).
    - 57 degrees is minimum hairpin melting temperature
    - Regular HiFi conditions
    - I currently have amplification settings at 57 degrees for 2 minutes.
  - Amplify from pZE21_A12C_T7RNAPolymerase riboregulated T7RNAPolymerase (already Mini-prepped) using primers tolC_A12C_F and RK24_A12C_R. ☑
    - PCR Conditions: Two Step 59 degrees, then 69 degrees for 3:30 seconds. (3500 bp)
    - Regular HiFi conditions
  - DPN1 Digest- Put in -20 freezer for TolC fragment ☑
  - DPN1 Digest for T7 RR fragment and put in -20 freezer ☑
  - Run Fragments on Gel. 140 V at 30 min. May need to play around with it ☑
  - Image- ☑
  - Gel Purify Fragments ☑
  - Nano-drop Fragments. If not enough for Gibson Assembly review process and primers for errors and redo. ☑
    - 101.647 for a12c
    - 24.058 for tolc

- Gibson Assembly
  - Gibson Assembly of amplified fragments above (riboregulated T7RNAPol and TolC from RK24). ☑
    - Produces linear fragment with overhang to bla site in RK24. It will replace Bla, introduce TolC, as well as introduce the T7 RNA Polymerase with its riboregulation system without interfering with the KorC gene.
  - Conditions:

Name of assembly fragments together

Frag	Size	%(by size)	ng/uL	ng/5	uL to add	Water
1	3300	0.647058824	101.647	20.3294	0.636574	
2	1800	0.352941176	24.058	4.8116	1.467043	
Total:	5100	1			2.103617	7.896383
						10uL total
Add 10uL of 2X NEB Gibson Master Mix to each						
Put in thermocycler - 50C for 60min, then 4C forever						

  - ☐
- **Pcr amplify gibson product** ☑

1) Using primers 245 and 247

- 3 min extension at 68 degrees- 5 kb was present, but too faint

2) Doing a New Approach:

1 PCR 6a	2 PCR 6b	3 PCR 6a	4 PCR 6b
2.5 uL DMSO, 19.5 uL H20	2.5 uL DMSO, 19.5 uL H20	no DMSO	no DMSO
68 degrees, 4 min ext	60 to 68, 4 min ext	60, 4 min ext	60 to 68, 4 min ext

- Still only getting a faint band,

3) Ordering shorter primers ☑

- using primers 250 and 251
  - 58 annealing
  - 5 min ext time
  - #1 PCR Tube= no DMSO (this worked)
  - #2 PCR Tube= DMSO (no band showed up)
- Run the Gibson assembled tolC and T7RR ☑
  - Run a gel- see a band at around 5 kb ☑
    - Protocol:
    - 140 V at 25 min.
    - Then, 130 V at 15 min.
  -
    - 1) Only 4.066 ng/uL
    - 2) Run 4 of them same conditions.- **Didn't work**
      - Elute with 50 uL of water for 4 gel fragments
      - PCR purify 2 and elute with 50 uL of water
      - Ideally have about 30-50 ng/uL
    - 3) Re-did Gibson with new Gibson Mix, Working Primers, and Nuclease Free Water. Then, will amplify with PCR HiFi again- **This worked**
    - 4) Amplifying TolC-T7RR fragment of 4.066 ng/uL. Will run on Gel- **Scrapped this**
- Image ☑
- Gel purify ☑
- Nanodrop ☑ - highest yield was 17.28
- Drop dialyzing for an hour ☐

- Talk to Natalie about next step- Recombineering
- Recombineer with linear fragment produced by Gibson Assembly. (T7RR-TolC).
- Strains from Natalie needed: 559 and 131
  - Transformation ☑
  - Conjugation ☑
  - Check plates ☐
  - Congrats, you're done!

T7 Riboregulation GFP Assay

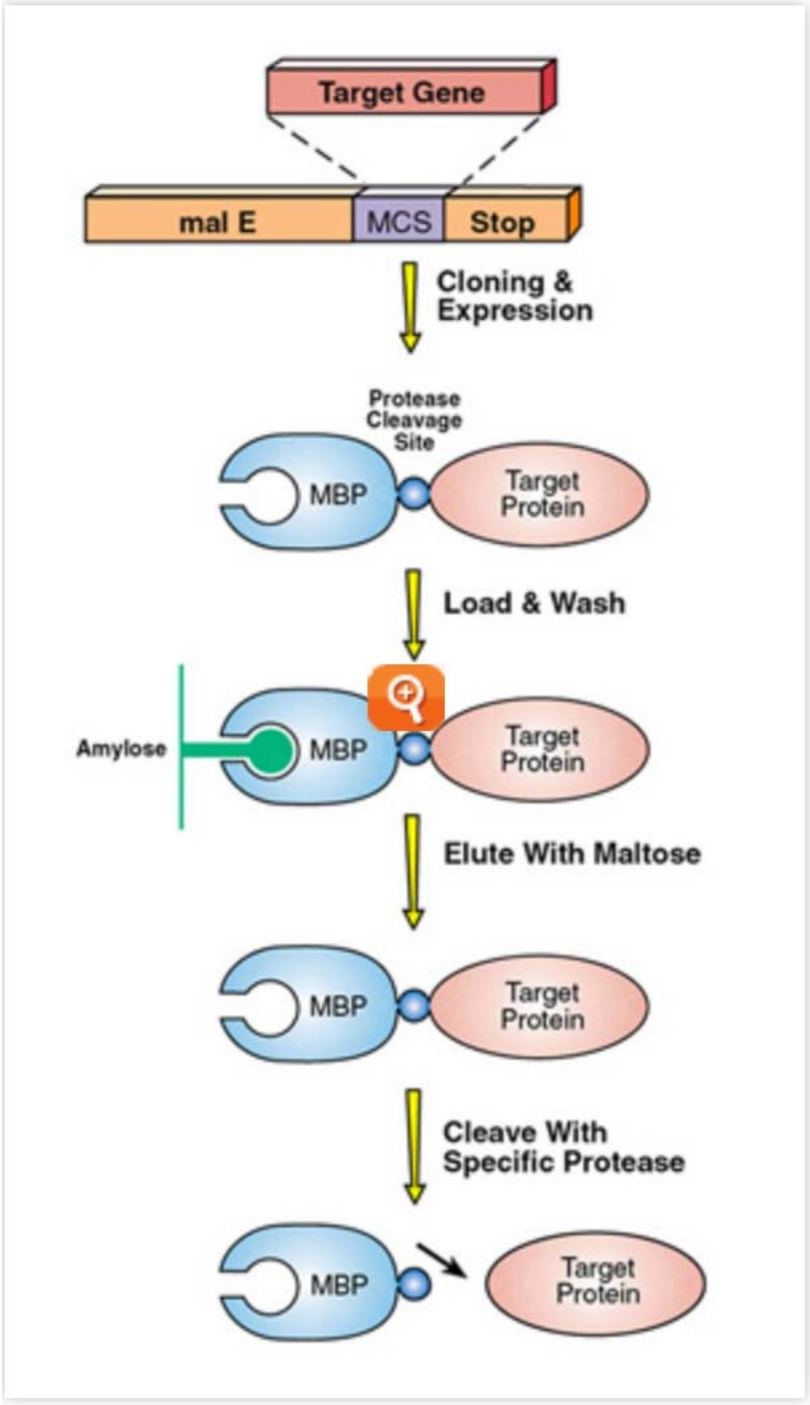
Author: stephanie_mao

Parts to construct: ECNR2+pZE21_A12C_T7RNApol+pZA21_T7sfGFP

How to get there: Transform ECNR2+pZE21_A12C_T7RNApol with pZA21_T7sfGFP, or transform ECNR2+pZA21_T7sfGFP with pZE21_A12C_T7RNApol.

Protein Purification Ideas

- Two approaches:
  - Use big tags like GST, MBP, or SUMO
  - Or go for the smaller tags like Flag, Strep, His, and Myc
- Use Maltose Binding Protein (MBP)
  - DOPA most likely to polysaccharides: downside
  - gene of interest needs to be cloned in a pMAL vector
  - the Ptac promoter will initiation expression of our target protein
  - system will fuse target protein with MBP that can then be isolated with amylose affinity chromatography
  - Disadvantage:
    - 27 to 396 amino acids in Tag
  - For more information, see https://www.neb.com/applications/protein-expression-and-purification/coupled-protein-expression-and-purification/maltose-binding-protein-expression



- Strep Tag
  - Tag stays attached to the target protein, so it is not the best option
- Sumo Tag
  - Only 8 amino acids
  - SUMO with a 6His that is removable
- FLAG Tag
  - Advantages:
    - fused protein can be easily cleaved by enterokinase
    - FLAG tag is hydrophilic so won't interfere with protein folding and functional—how are we sure about that?
    - M2 monoclonal antibody to FLAG is bound to agarose
    - only 8 amino acids
- His Tag
  - The protein will be permanently stuck on the protein we make
- GST Tag (Gluthathione S-transferase)

- 211 amino acid protein (26 kDa)
- fused to N-terminus of the recombinant protein
- Since GST rapidly folds into stable and soluble protein after translation, in promotes more expression of the recombinant protein
- purified by binding to its substrate (GSH)- glutathione
- To get rid of the GST tag, a protease can cleave between the recombinant protein and the GST tag
- This is what Natasha mainly uses in the Rinehart lab, and she says it is not that messy
- Downside: leaves 2 amino acids, a Glycine and a Serine

- Myc Tag
  - used for mammalian recombinant protein expression, so may not be applicable for us
- ELP Tag
  - ELPs have a reversible phase transition when they are heated above their critical solution temperature, so we can use this to our advantage.
  - Fuse our protein to ELP and cycle the solution through insoluble and soluble phases of ELP- Inverse Transition Cycling
  - Advantage: does not involve chromatography, cost effective
  - Disadvantage: We don't know how our protein will behave when we increase/decrease the temp/salt
  - Purification Method without any Tag:
  - http://www.microbialcellfactories.com/content/13/1/52
  - 1. CaCl2/EDTA treated whole cells + Isolated Inclusion Body
  - Centrifuge to get cell pellets containing MAP
  - use a washing solution (100 mM Tris–HCl (pH 8.0), 5 mM $CaCl_2$ in 100 mM Tris–HCl (pH 8.0), and 10 mM EDTA (Sigma) in 100 mM Tris–HCl (pH 8.0).
  - Resuspend cell pellets in 15 mL lysis buffer (10 mM Tris–HCl and 100 mM sodium phosphate; pH 8.0)
  - Cells should be lysed with the constant cell-disruption system (Constant Systems, Northants, England)
  - Centrifuge cell lysates, and cell debris with inclusion body should be collected
  - 2. Triton X-114 double washing of inclusion body + Acetic Acid Wash
  - treat inclusion body with Triton X-114 washing twice
    - suspended in Triton X-114 washing buffer (1% (v/v) Triton X-114 (Sigma), 1 mM EDTA, and 50 mM Tris–HCl; pH 8.0 and with 10 mmol phenylmethanesulfonyl fluoride (PMSF; Sigma) and 1 mg/mL lysozyme (Bio Basic Canada Inc., Ontario, Canada)
    - agitate overnight
    - after centrifugation, inclusion bodies responded in Triton X-114 washing buffer
    - Resuspend again in 40 mL of 25% (v/v) acetic acid so that we can specifically extract MAP
    - centrifuge
    - collect supernatant
    - dialyze in distilled water with 0.1% (v/v) acetic acid
    - freeze dry
  - 3. Ion exchange chromatography purification
  - To further purify the recombinant MAP, use fast protein liquid chromatography (FPLC; GE Healthcare, Buckinghamshire, UK)
    - prepare samples by dissolving freeze dried and acid extracted MAP in binding buffer (20 mM sodium acetate; pH 4.0).
    - use a cationic exchange column (HiTrap SP XL; GE Healthcare) to separate fl-151 and the impurities through a gradient of elution buffer (20 mM sodium acetate and 2 M NaCl; pH 4.0)
  - protein recovery at each step can be quantified with the Bradford assay (Bio-Rad, Hercules, CA, USA)
  - 20.5% yield
  - Other options:
    - Try a pH gradient to elute DOPA for the peptide
    - Incorporating cut sites, such as proteases like enterokinase
    - Possibly a Flag tag cut site with enterokinase ($100-$200)

Talking Protein Tags With Dr. Isaacs
- Arginine worry - (lower yield (44%)
- Calmodulin-binding peptide
    - order two, hybridize together. Clone into vector. Could probably put this together via gibson assembly.
- StrepX2 + Flag best bet. Talk to Karl and Natasha.

Twin-Strep Column
http://www.iba-lifesciences.com/Twin_strep_tag_buffers_reagents_Products.html?page=2
http://www.iba-lifesciences.com/details/product/2-1121-012.html
http://www.iba-lifesciences.com/isotope/2/2-1121-012-Manual--PPI.pdf

(Twin-) *Strep*-tag® Purification Short Protocol

iba

Purification of (Twin-) *Strep*-tag® fusion proteins with *Strep*-Tactin® matrices

(Twin-) *Strep*-tag®/*Strep*-Tactin® affinity purification

The ***Strep*-tag®** purification system is based on the highly selective binding of engineered streptavidin, called Strep-Tactin®, to *Strep*-tag® II fusion proteins. This technology allows one-step purification of almost any recombinant protein under physiological conditions, thus preserving its biological activity. The *Strep*-tag® system can be used to purify functional *Strep*-tag® II proteins from any expression system including baculovirus, mammalian cells, yeast, and bacteria. Unique Strep-Tactin® affinity columns have been developed for this purpose and the corresponding operating protocol is described below. Strep-tag®/Strep-Tactin® affinity purification should not be performed discontinuously in batch mode which would result in significantly reduced protein purity and yield in comparison to column chromatography. Further, prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag. Because of its small size, *Strep*-tag® generally does not interfere with the biological activity of the fusion partner. Thus, removal of the tag becomes superfluous. Comprehensive reviews and scientific publications giving an overview of various *Strep*-tag® applications are listed at www.strep-tag.com.
The **Twin-Strep-tag®** is a dimeric version of the Strep-tag®II and therefore binds with the same selectivity to Strep-Tactin® but with a higher affinity. This higher affinity allows the purification of Twin-Strep-tagged proteins even from batch or cell culture supernatants with good yields. In addition the Twin-Strep-tag® tolerates higher amounts of detergents and salts in buffers compared to Strep-tag®II. Since the overall conditions for Strep-tag®II and Twin-Strep-tag® are the same, the following protocol can be used for both tags.

Short Protocol of the *Strep*-Tactin® chromatography cycle

Perform all operations at a temperature amenable to the stability of your recombinant protein (between 4 °C and 30 °C). To achieve optimal purification results, comply with the specified volumes and their ratios (column bed, washing volumes etc., see page 3). At low expression levels, increase applied cell extract volumes to take advantage of the column capacity, without changing other volumes.

For research use only

Last date of revision: October / 2012 Version: PRD 0308

www.strep-tag.com 1

iGEM Timeline

Author: stephanie_mao
Projects:

T7 Riboregulation System: In integration phase

- Genomic Integration: strains 730 and 731 currently being recombineered for tolC, will then replace tolC with T7RR and negatively select for its deletion.
    - Current status: will screen tolC on Monday, and hopefully recombineer in the afternoon. Will then wait another day for confirmation.
    - Further steps: screening after genomic integration (will take one day), transformation of DOPA OTS into #730 (will take two days for results)
    - Estimated time of completion: hopefully by the end of summer.
- RK24 Integration

- Parts: T7 Riboregulation system (no illegal restriction sites), T7prom+sfGFP (has EcoRI sites)

Construct:

- Expression:
- Purification:
- Characterization:

- Parts: Complete construct (Tags+LL37+FP151+sfGFP, no illegal restriction sites) and any modular combination.

Protocol Explanations

PCR Primer Prep
(Make sure primers are resuspended at 200X)

1. Label tubes with the primer number
2. Take nuclease-free water in 100 uL pipette and put in tube
3. 10 uL from small blue stock tube and put into corresponding clear tube- the same one water was put into
a. mix in and out
4. Label the tubes

Setting up the PCR Reaction

Hifi Recipe - scale up for a master mix.
1. 25 uL HiFi master mix
2. 22 uL nuclease-free water in tubes- mix.
3. 1 uL of forward primer
4. 1 uL of reverse primer
5. 1 uL of DNA
(30 seconds per 500bp)

Fast Recipe - Scale up for a master mix
1. 10 uL Fast Master Mix

2. 8 ul nuclease-free water - mix
3. 0.5 uL forward primer
4. 0.5 uL reverse primer
5. 1 uL DNA template

(30 seconds per 1000bp)

Putting the Dpn1 (the volume is always 50 uL)

1. 1 uL of DPN1 into tubes- mix in
2. Incubate tubes with the DPN-1. DPN1 digest protocol programmed on machines.

Making a 1%Gel

1. Obtain 1 gram agarose for every 100 mL TBE needed for gel. Standard gel is 150 mL TBE
2. Add agarose to Agarose flask. Add proper amount of TBE to TBE flask using TBE labeled graduated cylinder.
3. Mix well and set in microwave for 1 minute at a time. Take out and swirl with red mitt until fully dissolved.
4. Prepare Cast. Use compass to properly align. Use combs for wells.
5. Allow it time to cool in agarose flask, then transfer to either EtBr or gel green flask.
6. Add DNA stain. EtBr is 100,000X. SYBR SAFE is 10,000 X.
7. Pour into cast and allow to cool further for 20-30 minutes. Will harden into gel.

Running on Gel for Gel Electrophoresis for Cloning

Xylene Cyanol (500bp) loading dye. - short fragments
OrangeG (300 bp) loading dye. - long fragments
FAST - has its loading dye already mixed in.

1. Take out the gel.
2. Put gel in proper apparatus. Only one apparatus for gel green.
3. 5 uL of proper DNA Ladder.
4. Gel Purification: 5 uL in first well. 25 uL in second and third well to see how much of DNA is necessary. Screening: 5 uL is fine.
5. Repeat process for each samples.
6. 2.5 uL DNA ladder for comparison
7. 5 uL of DNA ladder on other side
8. place at around 140 V and hit run on plate for 15-30 minutes.
9. Image with machine.

DNA Purification from agarose:
(QIAGEN Gel Purification Prep Kit)

1. Slice out the DNA from the gel
2. 300 uL QG buffer to dissolve the agarose
3. Put 3 tubes in heat block for 10 min so all agarose dissolves
4. Shake each of the tubes
5. Put 3 purification columns in plate with holes (the holder)
6. If agarose has not dissolved, add 100 QG buffer and put back on heat block
7. 100 uL isopropyl alcohol in tubes with DNA strands. The alcohol keeps the DNA in the columns.
8. 1,000 uL (i.e. as much as possible) and put into chromatography tubes
9. vortex the tubes
10. empty the top of tubes
11. add remaining liquids in the tubes and repeat process until everything is in the tube
12. Add 500 uL QG buffer to 3 tubes.
13. Centrifuge
14. Discard flow-through
15. 750 uL buffer PE wash to columns tubes. This process allows for de-salting so that annealing is more stringent for annealing.
16. Wait 2 minutes.
17. centrifuge
18. discard flow-through
19. centrifuge to get rid of ethanol
20. get new micofuge tubes with lids to go under the column tubes and label them.
21. add 50- 100 uL nuclease-free water into 3 tubes. This will cause the DNA to elute to the bottom.
22. wait a min.
23. centrifuge with lid on for 1 min.
24. Find out how much DNA is in each tube with a nanodrop. By this point, the DNA and water should be in the bottom of the tube.

*All centrifuges are for 1 min.

Culturing Cells

1. Start the Bunsen burner
1. Get big tubes and label them.
2. 3 - 5 mL of LB. Add appropriate antibiotic selector.
3. Wipe down the pipette with ethanol
4. Open petri dish with colony and scrape a colony
5. Pipette up and down, and scrape around the sides of tube to remove colony.
6. Wipe down with ethanol before each colony insertion
7. Put tape on the tops as labeling
8. Put in 37 degree incubator

Preparing Frozen Stocks
(with flame)

1. 200 uL 80% glycerol- prevents crystal formation
2. Add 1 mL of liquid culture
3. Pipette both up and down to mix with glycerol

Dialyzing Process for Gibson Assembly

1. Take out dialysis filters
2. Put them on the surface of Milli-Q water in a petri dish. The water acts as a reservoir to pull away salts.
3. Add samples, only onto the filter
4. Wait for an hour
5. Move it to a small PCR tube if it won't be used until the next day

*Put on lid to prevent evaporation

Transformation - always have a control

1. Turn on Bunsen burner
2. Chill the MilliQ and the cuvettes
3. Determine the DNA value: (____ ng/uL) (uL)= (50 uL) (1 ng/uL)
4. Take diluted dot and put into two separate tubes
5. Have 3x tubes per transformation, plus 3 for control.
6. Put 1 mL LB in every third tube
7. Put nuclease-free water (49 uL) in every second tube with DNA (1 uL)
8. 1 mL of our own culture into the remaining tubes
9. Centrifuge the culture tubes- speed at 13500 for 1 min
10. Rinse with MilliQ water and re-pellet. There needs to be two water washes to ensure that you do not have a lot of salt concentration.
11. Extract out the water from the pellet (which contains the bacteria)- our culture
12. About 90 uL to transfer pellet and get DNA and put into the cuvette
13. Put cuvette in frog
14. Get recovery media ready, which is the LB
15. Press the red button to get the time constant
16. Pipette up and down in the cuvette with the recovery
17. Place the contents back into the recovery tube—This is the process of electroporation
18. Place in the incubator for an hour- Tape the tubes down horizontally in incubator
19. Include a positive control, which could be a plasmid with several dilutions or an old plasmid from a pervious Gibson reaction

***Another option is to take 1 uL from Gibson before drop dialyzing and use that in transformation**

***Settings for Electroporator:**

Press on the following numbers-
5
1
1
1800 V- voltage
25 uF- capacitance
200 ohms- resistance
Brown cuvettes have 1 mL gaps

Choosing colonies post-Gibson Assembly

1. Light Bunsen burner
2. Get a 96-well plate (only if there are lots of colonies, otherwise put in a large tube)
3. Take a huge glass pipette and take out LB (15 mL) in the trough, or just 3 mL
4. Add 15 uL Kanomycin into trough as a streak through, or just 3 uL
5. Pipette into first row of wells
6. Continue to next row – all the way down to bottom
7. Touch a colony and dab into well to inoculate. It is ok to touch the bottom of the well. Make sure for each well to touch a different colony.
8. Keep the lid on bottom of row to keep track of row you are on
9. Put a filter film so no evaporation occurs in the incubator, and then put the lid back on
10. Put tape on either end
11. Label: Gibson Assembly plasmid name

ECNR (name of strain)
Gibson Assembly of [pEZ21-mKATE-sfGFP]
Date
Sf stands for super folder. It means the GFP is a strain that is really good at folding. It will stay stably folded even at high temperatures.

12. Put in the plate holder gently
13. Leave to incubate overnight

Mini-Prep Kit for DNA Extraction

1. Take cultures that were made from yesterday
2. Use pipette and take out culture and put into the three Eppendorf tubes.
3. Label the tubes
4. Centrifuge for 3 min
5. Use pipette and get out everything except the pellet (which contains the bacteria that contains the plasmid)
6. Add in the remaining culture into Eppendorf tubes

We spun down half of the culture and are now putting in the other half.

7. Centrifuge for 1 min. with the lid
8. Take out the excess fluid and leave the pellet
9. P1 Buffer- degrades the bacteria. Pipette the buffer up and down to make sure we resuspend the pellet. This should be done for all the tubes. – Resuspension process.
10. P2 Buffer- Breaks cells open (open cell membrane). Add this to all the 3 tubes.
11. N3 Buffer- Neutralizes the reaction. This prevents the plasmid DNA from degrading. Note that the genomic DNA has already degraded. Do this for all the tubes.
12. Cenrifuge Eppendorf tubes for 10 minutes at the maximum speed.
13. Get the filter tubes and label the tubes.
14. Avoid chunky stuff from side after centrifuge. Use pipette and put contents into the **filter tube.** This is when I need the filter tube.

15. Centrifuge for 1 min.
16. Discard the flow through
17. Add 500 uL of PB Buffer. This is isopropyl alcohol for washing.
18. Centrifuge for 1 min.
19. Get rid of the waste from the spin.
20. Add the PE buffer to the 3 tubes. The PE buffer contains ethanol.
21. Centrifuge for 1 min.
22. Place the new Eppendorf tubes into the rack.
23. Discard the waste from the centrifuge
24. Spin down again for 1 minute to get rid of excess ethanol (second spin down)
25. Move microfuge tubes to the new column tubes and wait 1 minute
26. Add nuclease-free water to the tubes
27. Centrifuge for 1 min
28. Final product: DNA and water
29. Use the nanodrop to see how concentrated the plasmid is

How to use Nanodrop

1. Turn on program: Take3, Nucleic Acid Quantification. Put the dots in towards/closest to the machine.
2. Color in the top 4 dots
3. Put 4 dots of nuclease-free water (usually needs to be 2 uL)
4. Put the plate into the reader and read it
5. Wipe away the sample points
6. Put 3 dots from the DNA/water tubes into the dot places
7. Put into the reader. Hit approve. Hit read.
8. Save the sample results to Excel.

MAGE (and recombineering) Steps

1. Get samples out from incubator during mid log phase
2. Now, we can induce expression of lambda cassette by putting in the shaker machine for 15 minutes.
3. Get ice
4. Put water and controls and experimental tubes in ice box
5. Prepping DNA:

a. Controls: get only water (make sure lid stays closed for the water)
b. Experimental: 49 uL water, 1 uL DNA

6. Place samples immediately in ice
7. Prepare LB recovery media (not iced)
8. Carry out transformation electroporation in the cold room
9. The recovery time is 2 hours

Plating

1. Put 50 uL onto the petri dish
2. Put beads on and mix around
3. Put into the incubator overnight

Gibson Assembly Screening

1.15 mL media
- LB= allows the cells to grow
-KAN= makes sure the plasmids are present
ATC (Anhydrous Tetracycline)= induces expression of the gene,
2. Get a 96-welll plate
3. Use a multi-channel pipette and put media into the 96-well plate
4. Put film and stir on shaker to resuspend the cells (from the Gibson Assembly results)
5. Move Gibson Assembly products with pipette to the media plate
6. Label the plate
7. Put on a film
8. Tape both sides
9. Put into new plate into the incubator.
10. The Gibson assembly results go in fridge.

*For the ancestry cell, there will not be any mKATE fluorescence. It will only be green, and this is a control to make sure that KAN or ATC is not failing. We want to see how much it auto fluoresces red without the RFP.

Reading the Fluorescence

1. Make PBS buffer
2. Transfer fluorescence results to a new plate
3. Put water in a new plate as a balance
4. Spin down in machine for 8-10 min.
5. Resuspend in PBS
6. Dilute in the black plate with PBS
7. Read the plate: Amplify first construct with PCR

PCR Purification

1. Get mico elution tubes
2. Have your PCR tubes
3. Add PB buffer to tubes
4. Add DNA target sequences to tubes
5. Centrifuge for 1 minute at the max speed
6. Dump out the excess
7. Add buffer PE
8. Centrifuge 1 min max speed
9. Dump out the excess

10. Centrifuge again
11. Add water
12. Put lids on inside and centrifuge
13. Do the nanodrop procedure

Gibson Assembly (Always need a total of 20 uL)

1. Small tubes with the plasmid and the gene of interest (T7)
2. Add water to these tubes
3. Get Gibson master mix and put in tubes- 10 uL
4. Put in the Thermal Cycler

Streak Cells:

Purpose: Allows bacteria to reproduce in the cell culture

1. First flame pipette tip and then let it cool in the agar before touching colony to prevent from killing the bacterial colony.
2. Using a pipette tip, scrape of a bacterial colony from the agar plate
3. Using a new agar plate, make a line near the top of the plate with the pipette tip
4. Make another line in the opposite direction with the pipette tip
5. Make a zig-zag pattern as a continuation from the previous step without lifting up the pipette

Making Agarose Gel

1. TBE (150 mL)- Nalgene brand
2. Agarose-1.5 g
3. Warm up for 1 min 30 sec.
4. May need to warm up again to get rid of the granules
5. Use about 15 uL of EtBR or GelGreen
6. Put on plate with the teeth
7. Check if it is balanced with the device
8. Pour the agarose and TBE (Make sure it isn't too hot)
9. Check the gel to see when it ceases being a liquid (about 15 minutes)
10. Put gel on the "No EtBr" plate

Making Antibiotics

1. Put in the Kan powder
2. Serological pipette- under 10 mL of water (or the specific solvent we need) and put into the conical green tube
3. Shake the contents in the tube
4. Get a syringe with filter- only use when fire is on
5. Put Kan through top of tube and transfer to each of the small microtubes. Throw the syringe in the sharps bin.
6. Even out volumes in the microcentrifuge with a pipette
7. Label the tubes
8. Place in the freezer box
9. Then, swirl antibiotic in LB media

Plating

1. For the time constants that are good, just plate with 50 uL
2. For everything else, spin it down and plate everything
3. The control needs to have the largest value, so if you had one plate where you plated everything then you would need to do this for the control
4. To plate all, spin down the recovery media and remove everything except 100 uL, and then take that amount and plate.

Restriction Digest

- For microgram of Template DNA (1000 ng)
    - 4 microliters buffer
    - 4 micro liters of enzyme TOTAL
    - Fill rest with water to 40 microliters total.

Ligation Reaction

- 5 ul of QuickLigase buffer
- 50ng total of vector
- 3:1 molar excess of insert
- 1 ul Quick Ligase
- 10 ul total reaction
- let the reaction proceed at room temp for 5 min
- drop dialyze

Florescence Activity Assay

- Grow 1 mL culture with selection marker and inducer overnight.
- Transfer 150 uL culture into V-bottom plate well.
- Use plate spinner on setting 0, temperature 24-25, and time 4 minutes. The plate shaker will pellet the cells in the v-bottom well over 4 minutes. It's a big plate centrifuge basically.
- Remove the supernatant (the media). Resuspend in 150 uL 1X PBS. Stock PBS is 10X.
- Transfer 150uL of culture from V-bottom plate to clear-bottom-plate-reader plate. Clear bottom plates are found in a bag in the flat-bottom plates cabinet near Adrian's bench. They are black with clear lids and clear well-bottoms. Do not mistake them for lid-less all black plates. They are not the same.
- Take clear-bottom plate with culture in PBS to plate reader. Start New Experiment.
- You must first measure the fluorescence activity. Use Mira's program. (will get the actual name in a bit). Save in an excel spreadsheet.
- Next, measure the optic density (will get name of program in a bit). Save results in the same excel spreadsheet.
- Calculate expression of GFP by dividing fluorescence activity by optical density (this normalizes the activity by number of cells).

- If for either program a well reports overload, what you must do is return 120 uL of culture to the v-bottom plate and add 120 uL of PBS to cultures in clear-bottom plate (this is a 1/5 dilution).

Meeting With Natalie

Map
Author: Ariel Hernandez-Leyva

Conjugation
- Horizontal Gene transfer is gene transfer between organisms in the same generation.
  3 Ways this happens
    - Transformation - Plasmid Transfer
    - Transduction - Viral infection
    - Conjugation - Transfer of genes directly between two cells
      - Dependent on conjugative plasmids (selfish genetic elements)
      - Cells with conjugated plasmid are F+ and express a fertility factor. When they find a F- cell the sex pillus latches onto the cell and reels it in like a fishing rod, dragging the cells directly together. Have some sort of direct connection that allows transfers of a plasmid to the new cell, converting F- cell to F+ cell.
      - Some are species specific. Others are very promiscuous. RK24 has very efficient transfer. Controlled by tra genes. (Don't fuck with these genes).
      - Needs an origin of replication (OriR) - Species specific OrI. RK24 is originally a pseudomonas plasmid. To move it you need OriT - an origin of transfer. Also needs a selection factor, selective advantages. Can be metabolic, resistance, reservoirs for resistance, toxin-antitoxin systems. Have a toxin and anti-toxin molecule, the toxin lasts longer than the anti-toxin.
      - When conjugating, you must have a way to kill the donor.

- Vertical Gene transfer (Standard Gene Transfer) is from parent to offspring.
  -lambda phage and lysogeny. Injects DNA and goes dormant for a while. Virus can recognize the health of the cell, recombine into the genome, and waits until the cell is healthy enough to make replication worth it.
  -Isolated lambda REDCI857
    - 1960s at 37+degrees it immediately enters the lytic cycle.
    - 2000s some guys what to recombine what they want into the genome. E. Coli pretty much sucked at this. Went back to this phage and pulled out several proteins. Lambda gam, exo, beta, and CI857. Allow recombination to happen. If a cell has the lamdbaRED cassette integrated into its genome. When you shift to 42 degrees, beta inhibitor breaks down and the other three proteins are immediately expressed. Add dsDNA that you want to insert into cell. In cell there is a recBCD complex that degrades foreign double stranded DNA. Gam inhibits this. Exo takes the ends of DNA and chews them back to create single stranded ends, beta then coats the single ends to prohibit secondary structure and guide recombination to a site within the genome. We always recombineer with selectable markers, because otherwise there are too many cells to screen.

MAGE
- Takes advantage of the lambda red recombination system.
- You can put in a ton of single stranded oligos into the genome and cause a variety of mutations. Don't need gam or exo, just needs beta and CI857.
Cage

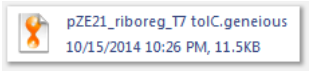
T7 Riboregulation Genome Integration

Author: stephanie_mao

Goal: To integrate T7 RNA polymerase with riboregulatory crRNA and taRNA into the genome of Natalie's strain 730.

Steps:
1. Replace Spe resistance in genome with tolC (Spe initially used to knock out pRFA-allows us to use DOPA OTS in strain)
2. Knock out tolC with a short oligo. (we could TRY knocking it out with the construct, but tolC is short and the construct is long and unwieldy).
3. We'll need to combine T7_RR with tolC in one of two ways:
   a. replace Kan with tolC, then amplify the whole shebang
   b. amplify up tolC and T7RR up separately, with primers that have homology to each other, and then GA them together.
   c. ACTUALLY, we're going to just place tolC in a genomic hotspot and then knock it out with our construct.
4. place the T7 riboregulation system into one of the genome's "hot spots," using tolC as the selectable marker.

Constructs:



Lab Notebook: T7 Riboregulation System

Map
Author: Ariel Hernandez-Leyva

Goal: Create a construct that incorporates T7 RNA polymerase in combination with a cis-repressing and trans-activating RNA strand to prohibit expression. Ultimately this system can be inserted into Genome.

Parts: pZE21 (PZE-CAT) plasmid backbone with cis-repressing and trans-activating RNA obtained from Ryan. BL21 DE3 strain containing T7 RNA polymerase obtained from previous team stock (Strain 5).

Lab Notebook

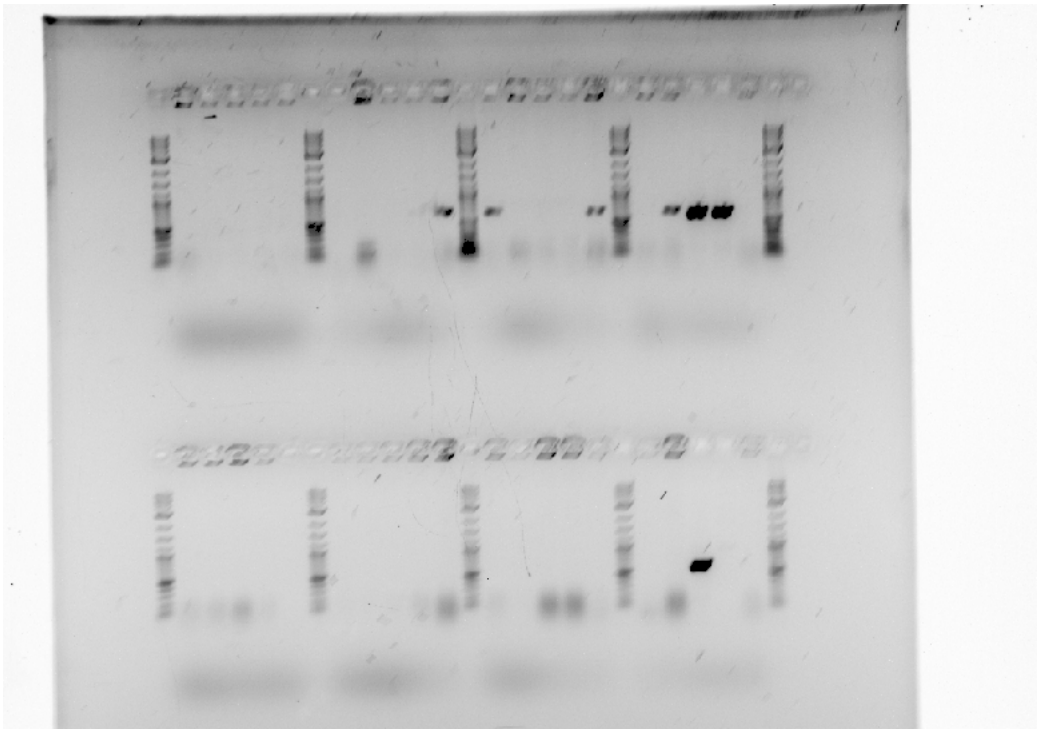
- Make the Parts
  - pZE21 backbone was amplified using primers 193 and 195 with overhang to T7 RNA polymerase.
    - DPN1 Digest
    - Gel Purification.
  - T7 RNA polymerase was amplified using primers 191 and 192 as well as pair 191 and 194. The two sets of primers both function, however one has overhangs and the other does not.
    - DPN1 Digest
    - Gel Purification
- Gibson Assemble
  - T7 RNA polymerase and pZE21 backbone were put together using Gibson Assembly.
- Transform into ECNR2
  - Drop Dialysis of post-Gibson Assembly Mixtures
  - Transformed into ECNR2 and plated on Kanamycin.
  - 4 Colonies grew successfully. Were inoculated into liquid culture. Frozen stock was made.
- Screening Construct
  - From Frozen stock (strains 82 through 85) were screened using sequencing primers and primers for T7 RNA polymerase and gel electrophoresis. Awaiting Results of Gel.
    - Results for First Screening with Ariel's protocol were not promising. There seems to be something happening in strain 85. However, the negative control appears to have a band of similar size?
- Incorporate into Genome of E.Coli

7/09/14

Tested strains 82-85 with internal to T7 Primers.

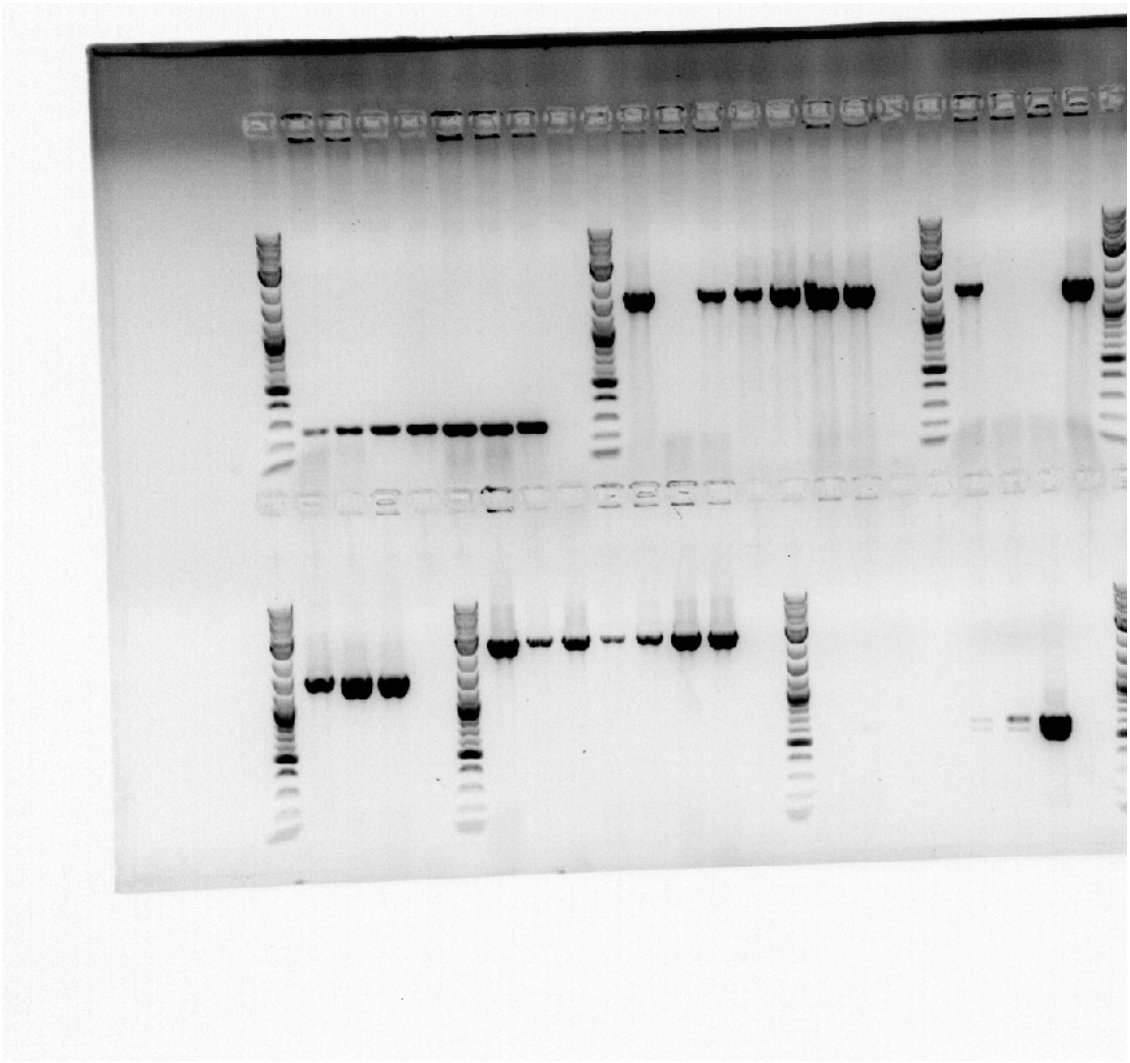
This will be our new screening protocol. Use primers 39S and 40S. Annealing temp 58 degrees, extension time 20 seconds. Positive Control: BL21 DE3. Negative Control: pZE-CAT or BL21 if none is available.

Strains 82, 83, 84, and 85 do not contain T7 RNA polymerase gene.

- Screened 17 Colonies for T7 RNA polymerase
  - Prepared 50 microliter micro titers of colonies.
  - Transferred 1 microliter colony titer to KAPA2G PCR mix with T7 RNA Pol Internal Primers - 39S and 40S
  - Ran PCR at 58 dC annealing temp for 20 seconds extension.
  - Ran Gel for PCR products for 17 colonies with positive control (BL21DE3) and negative control(BL21) at 140Volts for 15 minutes.
  - Imaged Gel
  - 16 out of 17 colonies contain T7 RNA Polymerase Gene
- Second Level of Screening
  - Transferred micro titers to 96 well plate. Also transferred fresh colonies to 96 well plate. 150 micro liters per well with 3 micro liters kanamycin + 1 micro liter cell.
  - Covered 96 Well Plate with paper cover, label, and stored in Isaacs Lab incubator and shaker overnight to gro
  - Repicked colonies. There might not be a positive control available—we know that both the internal and external primers work, so I think we're ok.
  - Control Grew
  - Repicked Colonies and added to 15 microliter micro titer LB+Kan for second level of screening
  - Set up 2 KAPA 2G Fast Reactions with positive control (PZE21:CAT with outside primers) and negative Control (Mach 1 Cells and PZECAT with primer fragments). Ran PCR at 58 degrees for 1:10 seconds each
  - Run on a SYBR SAFE GEL (4 micro liters) for 30 minutes at 140 Volts. Gel Imaged.
  - 

- Nothing above 1 kb appears. Clones seem to have failed.

- Screening from Colonies (7/14)
  - Restarted a 96 well plate from colonies on 7/11.
  - Made frozen stock of plate.
  - Screened 12 colonies with 5 different PCR reactions
    - Internal sequencing primers
    - 191 and 39S
    - 192 and 40S
    - 191 and 192
    - 37S and 38S
  - Run Gel of different PCR reactions.
  -



  - Samples 2, 5, 6 and 7 from Plate suggest presence of T7. Must be screened for connection to pZE21
- PCR Reaction for T7 RNA Pol - pZE21 backbone
  - Goal: Show T7 RNA Pol is inserted correctly into the pZE21 backbone.
  - Run 3 KAPA2G FAST reaction for samples 2, 5, 6, and 7 from plate in 4 dC fridge.
    - Use primer sets: (37S&38S, 37S&39S, and 38S$40S) with 5% DMSO
    - If this works, perform same reaction with HIFI w/ 5% DMSO. Submit to sequencing.
      - This PCR did not work.

Third Attempt from Bottom Up for T7 RNA Polymerase::pZE21 Backbone

- Obtaining Parts (7/10/14)
  - Took culture of BL21 DE3 with pZECAT transformed in and mini prepped.
  - Set up 4 HIFI PCR Reactions. Reaction 1 is HIFI plus primers for T7 RNA Pol. Reaction 2 is HIFI plus primers for pZECAT. Reaction 3 is HIFI plus DMSO plus primers for T7 RNA Pol. Reaction 4 is HIFI plus DMSO plus primers for pZECAT
  - PCR complete - added DPN1 for eliminating template DNA.
  - DPN1-Digest complete. Transferred to freezer until a SYBR SAFE Gel can be made.
  - Run on SYBR SAFE Gel for 30 minutes, at 140 Volts. Gel Imaged.
  - Well Defined Bands on Gel. Examined under blue light and excised bands. Placed in labeled tubes

- Gel Purified the DNA obtained and moved to labeled tubes. 50 micro-liters.
- Nano-dropped. Sufficient concentrations. (7/11/14)
- Gibson Assembled Parts.
- Drop Dialyzed.
- Transformed into Mach1 Cell culture. Plated on Kanamycin Plates.
  - Time Constant 1: 4.9s 2: 5.0s Control: 5.0s
  - Plated on kanamycin plates and left on bench over weekend to grow slowly.
  - Remainder of culture was returned to 4 degree refrigerator. Taped to wall.
- Screened 12 cultures.
  - Ran Kapa2G with internal primers.
  - Initial screening did not display any T7RNA polymerase.
  - Screened an additional 12 colonies

Sequencing Entire Plasmid (Second Gibson Attempt)

Goal: Due to the difficulty in obtaining a PCR product that can be sequenced we will be attempting to sequence the entire plasmid. In order to do this we need to amplify the plasmid via culture, obtain it via Mini-Prep, and then send it in to GenScript with a primer in order to sequence it.

7/15/2014

- Make YT Media to grow up a strong culture (2XYT 500 mL)
- Start cultures of 2, 5, 6, and 7 in YT media

Restriction Digest Assay of Plasmid (Second Gibson Attempt)

Goal: Due to the difficulty of obtaining a PCR product that can be sequenced we will be attempting to digest the plasmid and assay the fragment size via gel electrophoresis. In order to do this we need to first amplify the plasmid via culture, obtain it via Mini-Prep, and then conduct a restriction digest. To conduct the digest, we must determine the best restriction enzymes to use and learn the protocol for using them.

7/15/2014

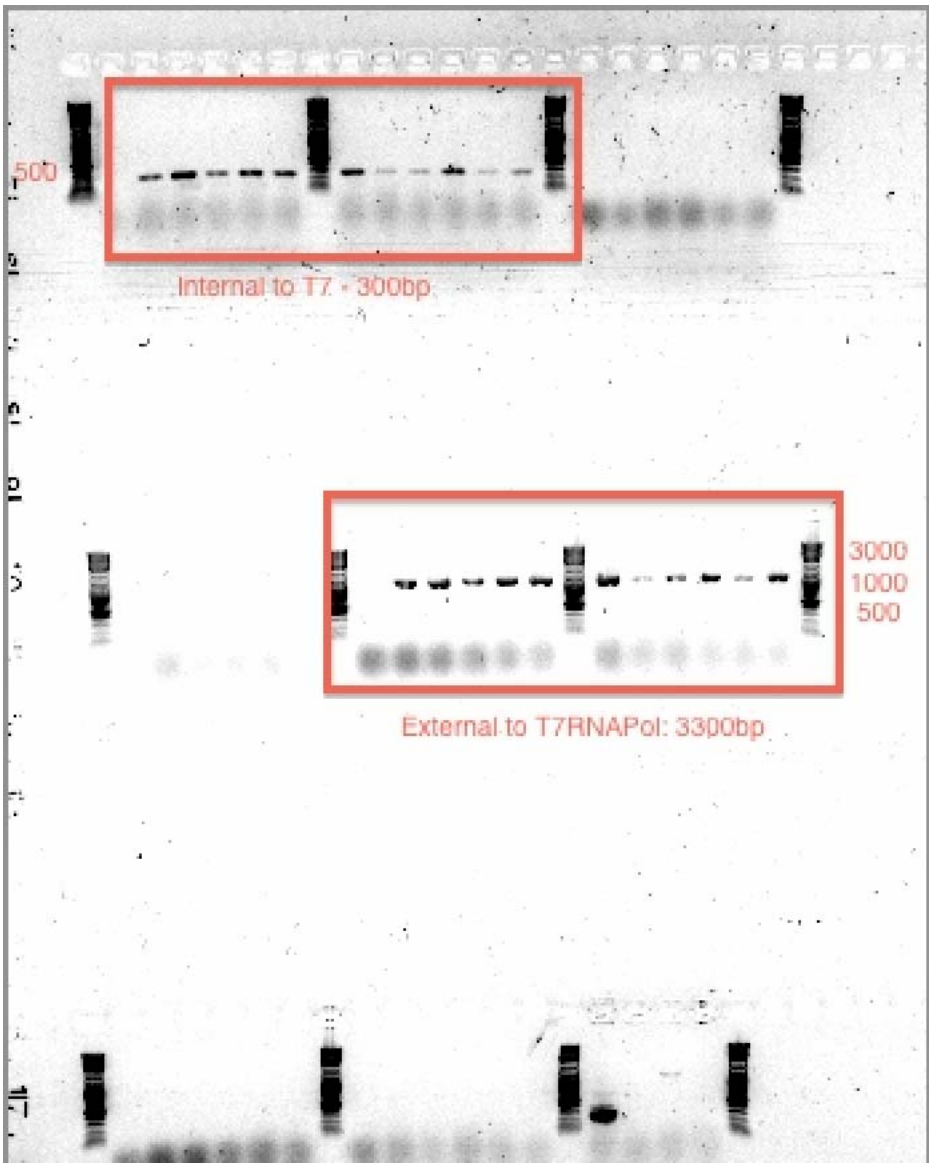
- Make YT Media to grow up a strong culture. (2XYT 500 mL)
- Started Cultures of Clones 2, 5, 6 and 7 in YT media

7/16/2014

- Mini-prepped cultures, but couldn't do restriction digest because we're out of pZE21+CAT, which means no negative control.
- TOMORROW will do restriction digest.

7/17/2014

- Performed Restriction Digest. Three rows. 1: Double Digest With ApaL1 and Xba1. 2: Single Digest Apa1 3: no restriction enzyme. Just plasmid.
  - Ran on Gel with orange g.
  - Imaged Gel
- Testing t7RNApol external promoters:
  - Ran PCR on pZECAT
  - Ryan's Primers should be used for everything. Mine suck. Don't use them.



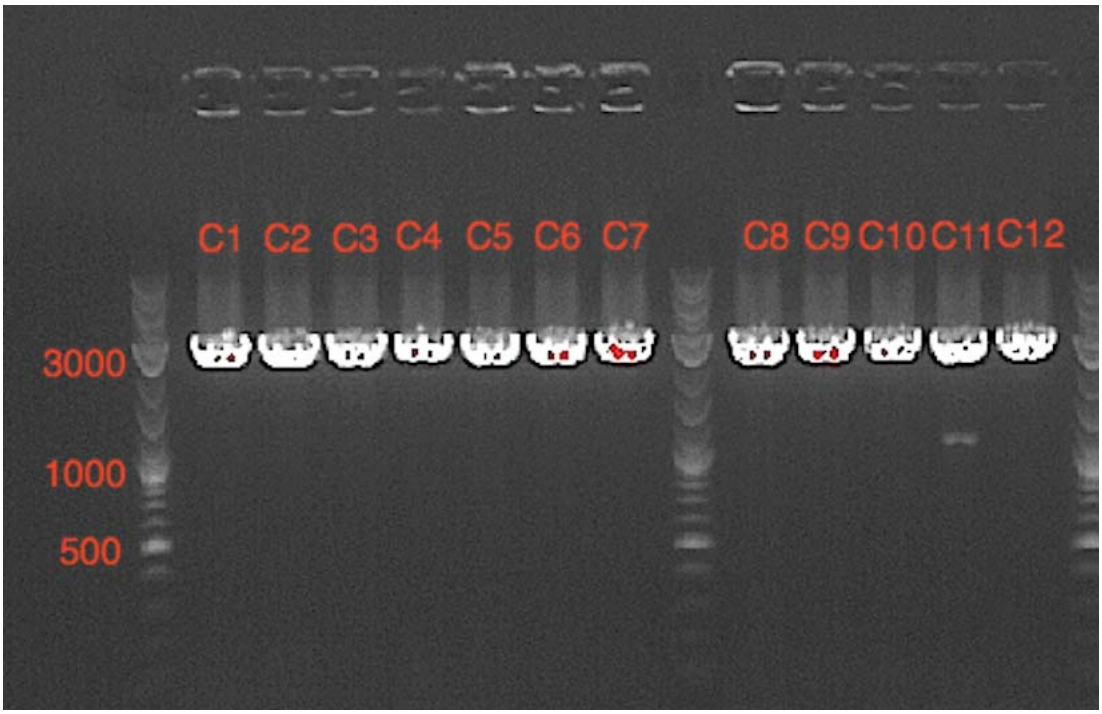
Results of Gel running with Ryan's Primers: 810 and 811 and Ariel's internal primers 39S and 40S

7/19/2014

- issues with primer 193: not correctly reverse complimented. Order for the correct primer will be placed on Keck. This will hopefully solve the problems that we have been facing.
- https://www.youtube.com/watch?v=i1EG-MKy4so which means the past few weeks have basically been this video.

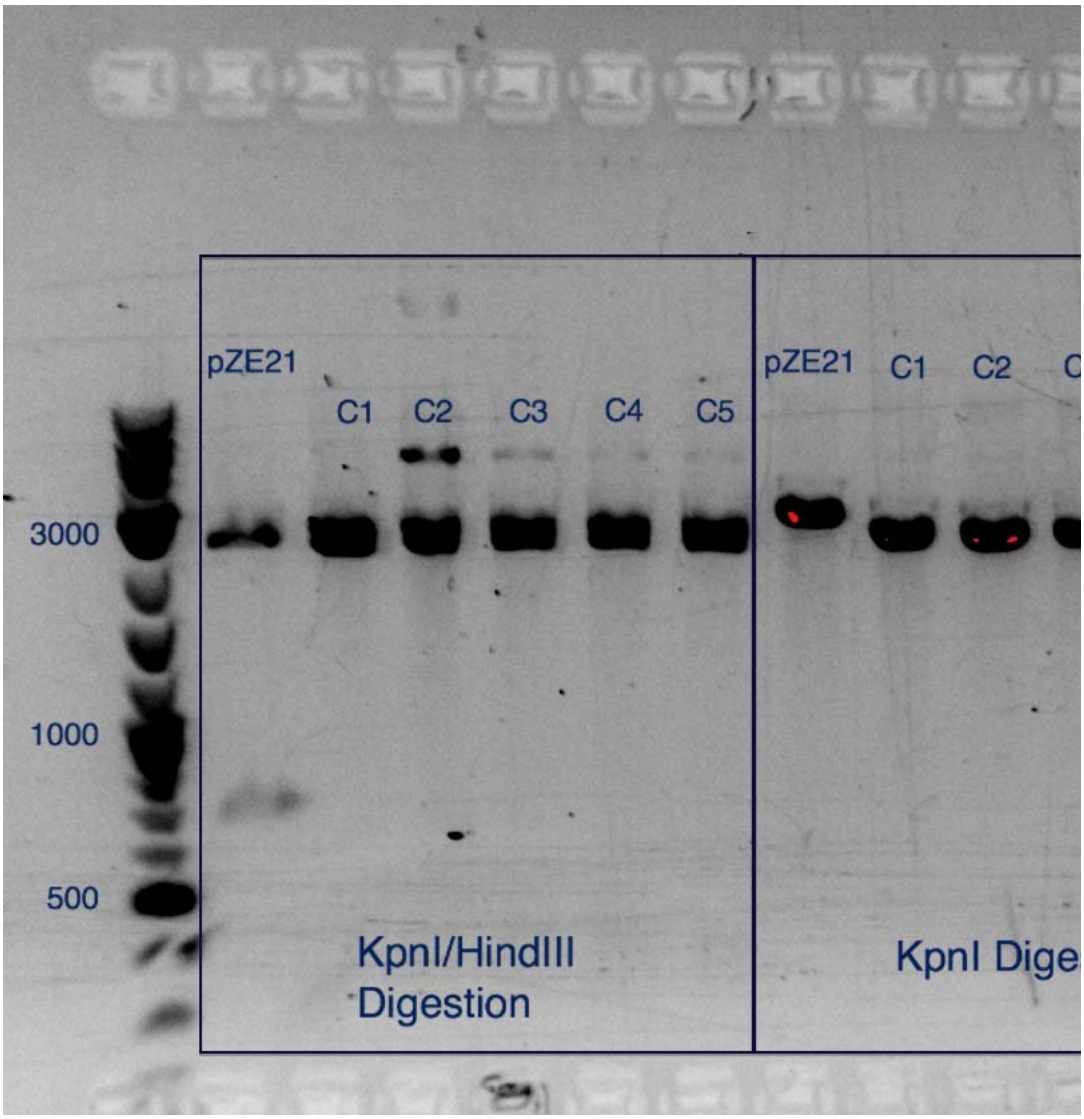
  - Edited Protocol for Assembly of T7RNAPol pZE21 ☐
    - Amplify T7RNAPol from BL21 with primers 191 and 192 ☐
    - Amplify pZE21 from pZECAT with primers 196 and 197 ☐
      - Need to order primer 196 ☑
    - DPN1 Digest template DNA. ☐
    - Run fragments on a gel green Gel ☐
    - Gel Purification of fragments. ☐
    - Gibson Assembly of fragments into plasmid construct. ☐
    - Drop Dialysis of Post Gibson Assembly products. ☐
    - Transform plasmid construct into Mach1 Strain (one with pZA21+T7GFP if we have that going by then). ☐
    - Plate Colonies on Kan (+Spe) Plates 1 hour later. ☐
    - Pick colonies from Kan (+Spe) Plates, inoculate liquid culture. ☐
    - From liquid culture make a frozen stock. ☐
      - simultaneous start a 96 well plate of potential clones. ☐
        - Can also test with clones in cloramphenicol. Should all die. ☐
    - Screen colonies for presence of plasmid of interest ☐
  - Screening Protocol for T7RNAPol::pZE21 ☐
    - From a 96 well plate start PCR reactions with sequencing primer pairs 39S&40S, 810S&811S, and 39S&810S, and 40S&811S. Use BL21DE3

and pZECAT as controls. ☐
    - Run Gel
        - 39/40 - 300 bp if T7RNAPol is present.
        - 810/811 - 3300 bp if T7RNApol is present. 1100 if CAT is present.
        - 39/810 - 2012 bp if T7RNApol is present,
        - 40/811 - 1613 bp i T7RNApol is present,
- Protocol for Assembly of T7RNAPol pZE21 with Jaymin's Primers
    - Amplify T7RNAPol with primers 193 and 194. ☑
    - Amplify pZE21 with Jaymin's primers 227 and 228/685. ☑
    - DPN1 Digest Template DNA ☑
    - Run Fragments on a gel green Gel ☑
    - Gel Purify fragments. ☑
        - for pZE21 made with 685 run restriction digest ☑
            - about 1 microgram of DNA (also ran 4 micrograms of DNA will collect and use for further Gibson Assemblies as Needed)
            - 4 uL cutsmart
            - 1 uL KPN1-HIFI
            - Rest with water to 40 micro liters
    - Gibson Assembly of fragments into plasmid construct. ☑
    - Drop Dialysis of Post-Gibson Assembly Products ☑
    - Transform post-gibson assembly products into Mach1. ☑
    - Plate colonies on Kan plates 1 hour later. ☑
    - Pick clones from Kan plates and inoculate liquid culture. ☑
        - make 96 well plate for frozen stock ☐
        - make 96 well plate with Kan ☑
        - make 96 well plate with CAT. Anything that survives the Cat plates should be excluded from the Kan plate clones. ☐
    - Screen Clones for plasmid of interest ☑
        - From a 96 well plate start PCR reactions with sequencing primer pairs 39S&40S, 810S&811S, and 39S&810S, and 40S&811S. Use BL21DE3 and pZECAT as controls. ☑
        - Run Gel ☑
            - 39/40 - 300 bp if T7RNAPol is present.
            - 810/811 - 3300 bp if T7RNApol is present. 1100 if CAT is present.
            - 39/810 - 2012 bp if T7RNApol is present
            - 40/811 - 1613 bp i T7RNApol is present
    - SUCCESS!!! WITH JAYMIN'S PROTOCOL!!!
    -

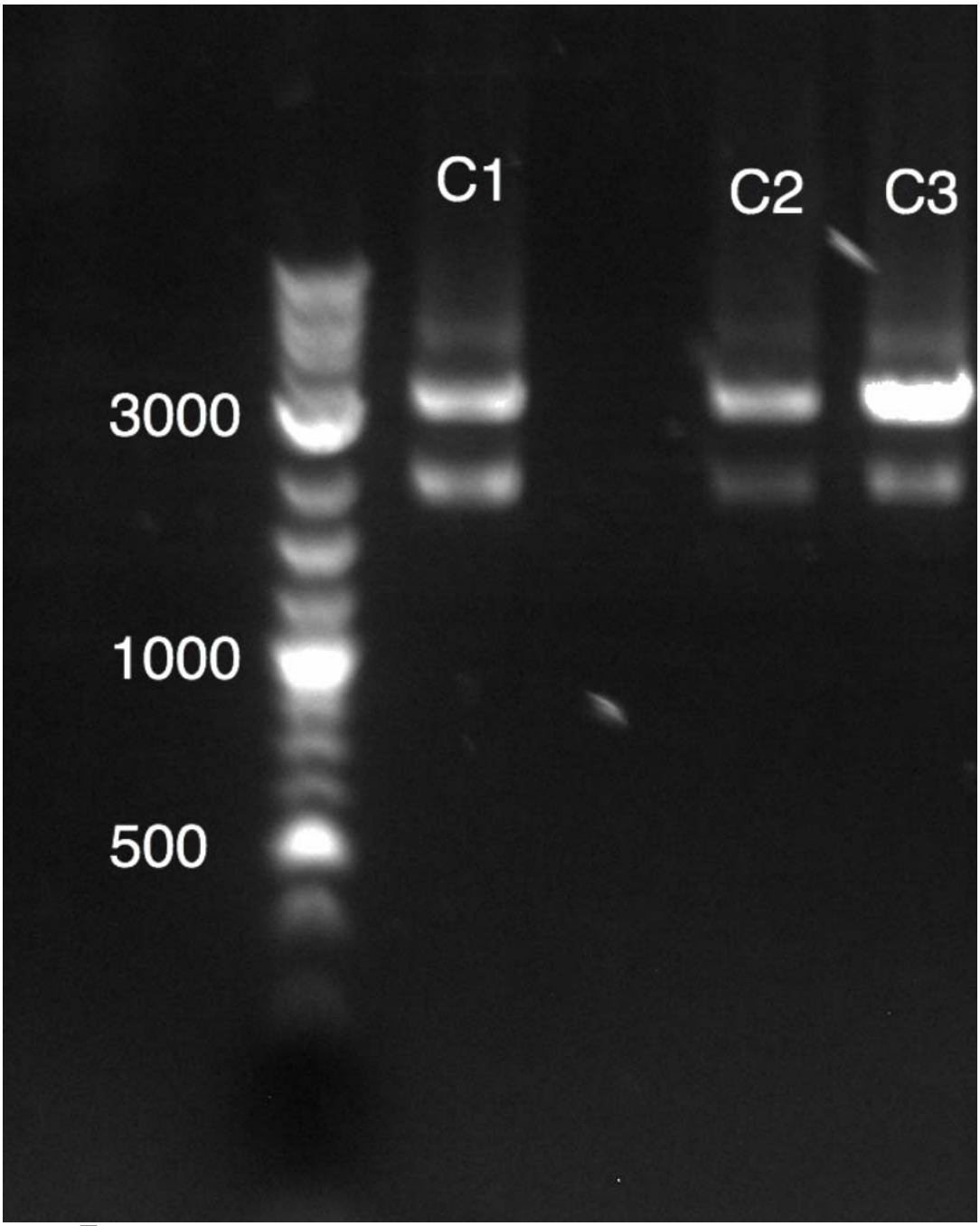


7/24/13

- Now that we think we have T7RNAPol we should start screening for it to be absolutely sure.
- DNA Screen ☐
    - Mini-prep first 5 clones of row C - started in YT culture in incubator upstairs and BL21DE3 w/ pZE21(CAT) ☑
    - Single Digest Assay ☑
        - Cut with XbaI and NdeI to compare with pZE21:CAT ☑
            - 300 ng of plasmid.
            - 1.5 uL Buffer
            - 0.75 uL enzyme
            - fill to 15 uL H2O
            - digest for 1 hour

- Xba1 is in backbone of pZE21 Cat
- Nde1 is in T7RNA polymerase
- PCR purify after digest. ☑
- Run gel electrophoresis (EtBr) on sample. Image. ☑
- 

- Sequencing ☐
  - PCR amplify using Ryan's Primers and HIFI. Send for sequencing to Keck. Follow Keck sequencing guidelines online. ☐
    - 500 - 600 ng DNA
    - 2 uL of 4 uM primer. Our primer is 20 uM. (dilute by 5). Only a forward/only a reverse.
    - Fill to 18 uL total volume.
    - Put it all in a Keck Sequencing tube.
    - Stephanie could do this and it would be great.
  -



- Functional Screen
  - Transform T7_sfGFP_pZA21 and T7RNAPol_pZE21 into ECNR2.
  - Express sfGFP and assay for GFP.

Sequencing:

T7RR Sequencing.geneious
10/15/2014 10:26 PM, 17.5KB

Our construct is messed up. We're going to clone the tacr system inside via restriction digest with Kpn1 and Xba1

Lab Notebook: T7 Promoter Plan 1

Map
Author: Ariel Hernandez-Leyva

Goal: Assemble the T7 promoter via primer overhangs on pZE21 with sfGFP, and then transform onto BL21 DE3 (which has the vector for T7 RNA pol expression) to screen.

Parts: pZE21 with sfGFP, two variations of the T7 promoter. (One was 'strong,' some initial research yielded no answer as to what that meant). BL21 DE3 strain.

Lab Notebook:

7/3/2014
Yamini plated the T7 promoter in BL21-DE3 on the Kan plates. Please check how they did tomorrow.
7/4/2014 Update: they did terribly.

7//9/2014
Transformed pZE21+sfGFP+promoters in Mach 1 strain and plated on Kan. Many colonies, however control also slightly positive. Will sequence and send in for screening (unfortunately there are no other quick and dirty methods of screening for this one.)

7/10/2014

- Screening for T7 Promoter by sequencing.
  - Picked Colonies fresh from successful T7 promoter plate and added to 50 micro liters LB+Kan - Ariel
  - PCR-amplified 10 colonies using FL13 sequencing primers Natalie let us borrow that are specific to pZE21, and ran them in gel, with Jaymin's plasmids as comparison. All successful, with colony #10 a little bit wonky. Used pZE21+sfGFP+linker as positive control (bright band, higher than others), and Mach 1 colony as negative control (worked). PCR purified samples 1-3, and will send them off to sequencing along with Jaymin's mini prepped T7 plasmid from earlier. And thus, plans 1 and 2-gamma-red may yield the locks for which we have no key...

7/11/2014

- Frozen stock of 5 successful T7 Promoter sequences. 3 sequences sent off for sequencing.

AND NOW: PLAN 2 GAMMA RED

Plasmid Jaymin gave us with pZE21+T7 promoter + gfp was transformed successfully into BL21 DE3. Frozen stock made, plasmids prepped.

7/16/2014

- Received sequences of T7 promoters. Jaymin's plasmid and all three samples seem to be in good shape.
- Will use FROZEN STOCK #1 as its sequence is the most clear (#2 has a deletion)

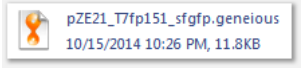
7/24/2014
Goal: we want to insert the T7 promoter with sfGFP into a new plasmid so we can co-harbor it in a plasmid with the T7 RNA Polymerase in a plasmid. Having similar sites of origin wouldn't allow this to happen. Therefore we are transferring T7 promoter with sfGFP into pZA21, p15A origin site, spectinomycin resistance.

- Ordered new primers 231 and 232 for T7_sfGFP. Will amplify with overhangs containing restriction sites for AatII and BamH1.
  - Protocol for amplification and assembly of T7_sfGFP_pZA21 ☐
    - YT culture of pZA21 growing in incubator upstairs. Must be mini-prepped in the morning. ☐
    - Mini-prep of T7_sfGFP-pZA21 must be amplified using primers 231 and 232 with 2-Step-HiFi PCR protocol ☑
      - 57 degree initial annealing temp. 69 degree initial annealing temp. ☑
      - 1:00 minute extension time. ☑
      - DPN1 Digest ☑
      - PCR purify (in fridge) ☐
    - Conduct Double Digest of 1000 ng of each Template DNA ☐
      - Add 4 uL buffer
      - Add 2 uL of each enzyme (AatII and BamHI)
      - Make up to 40 uL with dH2O
      - Run for 1 Hour at 37 degrees. Leave at 4 degrees afterwards.
    - PCR purify Double Digest contents. ☐
    - Run Ligation Reaction with template DNA.
      - 5 ul of QuickLigase buffer
      - 50 ng total of vector
      - 3:1 molar excess of insert
      - 1 ul Quick Ligase
      - Bring up to 10 ul total with H2O
      - let the reaction proceed at room temp for 5 min
    - Drop Dialyze
    - Transform into Mach1 and ECNR2 w/ T7RNAPol:pZE21tacR

7/29/2014
Successfully screened messy version of pZA21+T7sfGFP, and transformed it into ECNR2 with pZE21_A12C_T7pol (4 variations, to be exact).

Construct Designs

Author: stephanie_mao
Modular Format: Dopa Foot-Linker-AMP/other module



-
-

pZE21_T7LL37-li36-fp511.geneious
10/15/2014 10:26 PM, 9.6KB

Update 7/15: will be using fp151 majority of modular constructs
New Constructs:

Constructs 0715.geneious
10/15/2014 10:26 PM, 20.7KB

Status of constructs: soon to be ordered.

Update 7/17: Ordered constructs with Genscript

Update 7/21: Updated mefp_1 and linker to not be constantly repeating at the codon level.

Constructs 0721.geneious
10/15/2014 10:26 PM, 22.0KB

Physical format: Create “arches” and other structures with short DOPA-containing peptide sequences.

Update 7/24: why order two constructs when you can have a MEGA CONSTRUCT

pZE21_T7LL37-li36-fp151-sfGFP with tags.geneious
10/15/2014 10:26 PM, 11.5KB

Construct Design Comments from Natalie:
His tags
Labe T7 as a promoter
Find out if all the tyrosine's become L-DOPA
Deleting hydrophobic amino acids on LL-37
Determining if it matters what side of the mussel protein we add the linker on
Try out different combinations of Mefp (i.e. Mefp 151, Mefp 511)- Attach to LL-37 on the N-terminus
Add in antifouling peptide constructs
Put all sequences in JCAT (on the original sequence, not the TAG sequence) ☑
Updated mefp-1 to have different codons in its consensus sequence to avoid repetition when using primers.

Major Antifouling components:
See Antifouling page

De Novo Peptide Creation

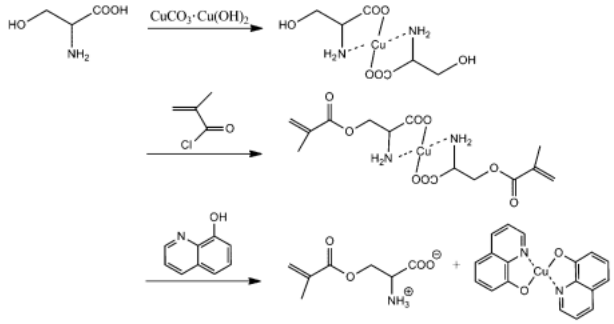
Things look slightly grim on our end because most of what we’re looking for in terms of antifouling are going to be difficult to simulate with only amino acids

Alpha Helices: Should be short, and have medium-size amino acids (no glycines, tryptophans, or pralines). Turns at about 3.6 amino acid groups, so the sequence should go N-Lys-Dopa-Lys, repeating. May potentially put in a different, neutral spiral at every other turn to minimize Lys charge.

Pillars: The foot sequence can be N-Pro-Lys-Dopa-Lys-Pro-N, with the Prolines acting as ways of inducing rigid structures.
Since not much is known about anti-fouling, we can experiment with different sets of residues to see what kind of surface—hydrophilic, hydrophobic, uncharged or charged.

Post Translational Modification
This paper uses serine as a basis for the generation of antifouling polymer serine methacrylate. We can create a scaffold of serines which then can be chemically modified to create different antifouling polymers.

Chemical synthesis of SerMA:



Not sure about how the first step with creating a chelated copper compound would work on a polypeptide amino acid instead of a zwitterion—could we skip it, as well as the final step, and just attach MA on via methacryloyl chloride.

Someone's thesis about the topic.

Protocols Ariel Collects

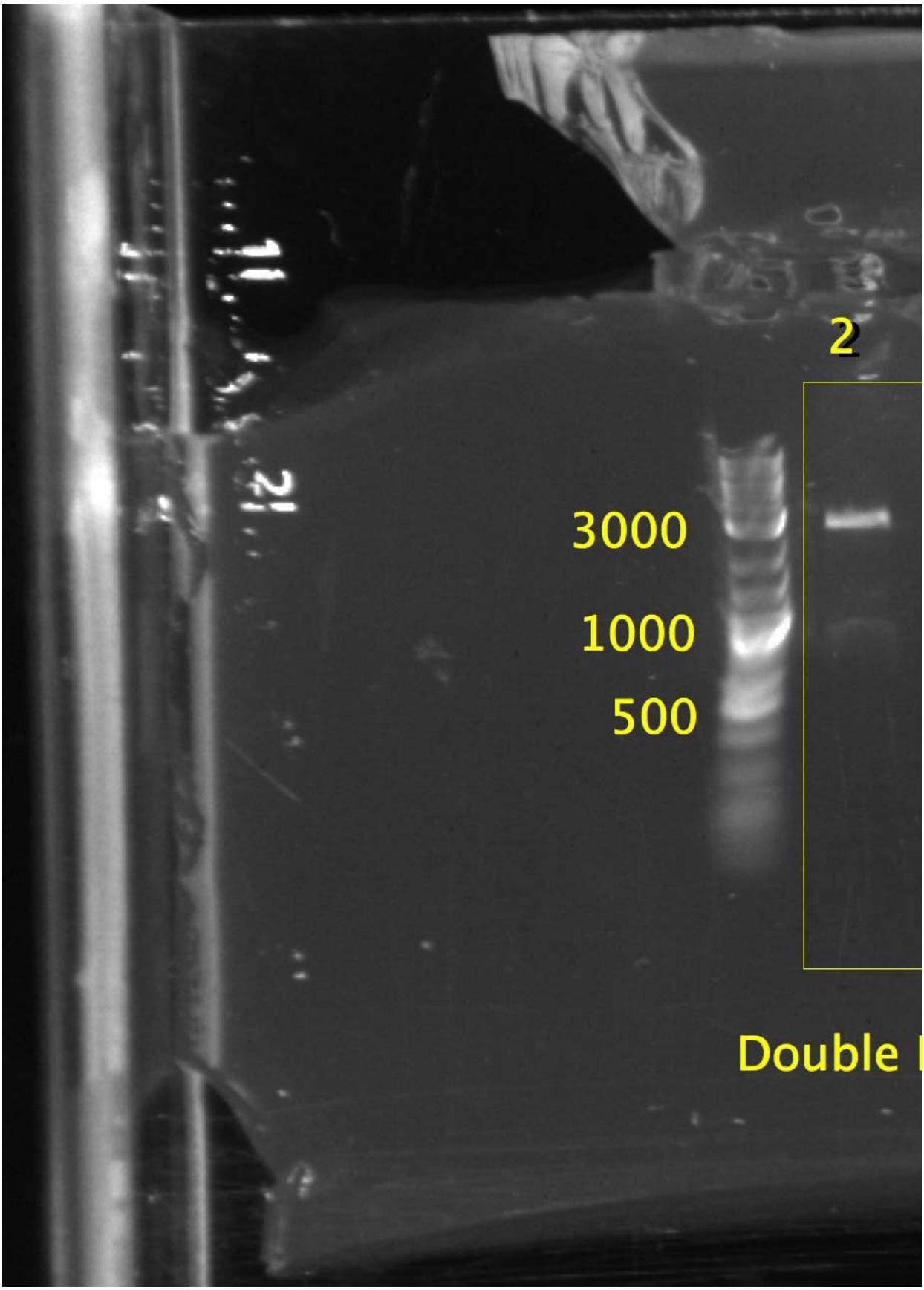
Map
Author: Ariel Hernandez-Leyva

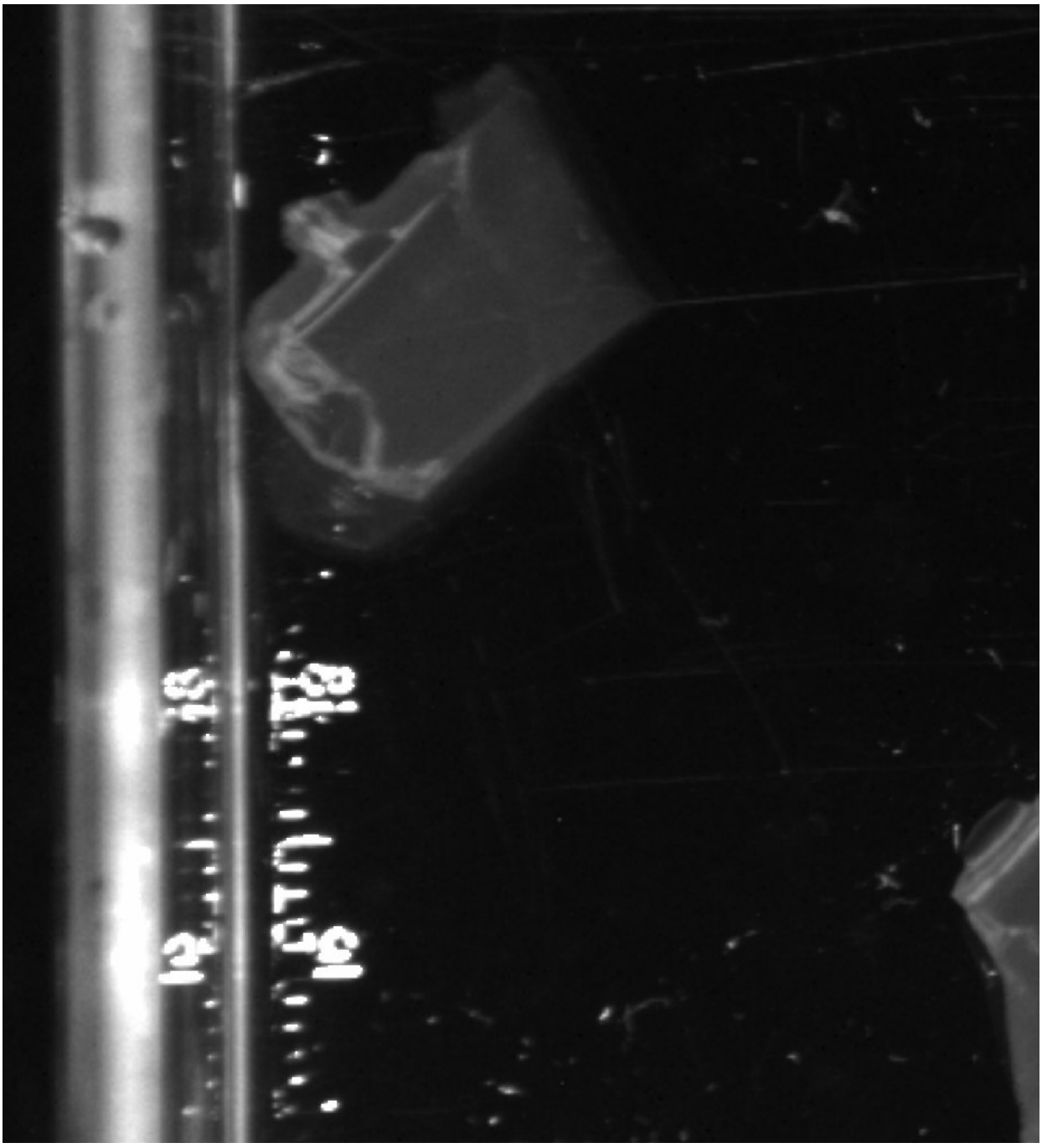
Making Frozen Stock 96 Well Plates:

- For half a plate (48 Wells)
  - 1 mL glycerol
  - 2.5 mL LB
  - 100 microliters mixture into a well. 50 micro liters culture.

Double Digest
Check https://www.neb.com/tools-and-resources/interactive-tools/double-digest-finder for the correct buffer.

- For microgram of Template DNA (1000 ng)
  - 4 microliters buffer
  - 2 microliter each enzyme
  - Fill rest with water to 40 microliters total.
- Dr. Isaacs Double Digest (300 ng DNA)
  - 1.5 uL buffer
  - 0.75 uL enzyme 1 (sum of enzymes is 10% Total reaction Mixture)
  - 0.75 uL enzyme 2
  - 300 ng template DNA
  - Make up with nuclease free water to 15 uL
- Dr. Isaacs Single Digest (300 ng DNA)
  - 1.5 uL buffer
  - 0.75 uL enzyme (5% total reaction mixture)
  - 300 ng template DNA
  - Make up with nuclease free water to 15 uL
- Restriction Digest protocol for 7/18/2014 - Restriction Digest Assay
  - Four Samples and Control (pZE:CAT)
    - Miniprep all clones potentially carrying pZE21::T7RNAPol
    - Nanodrop for concentrations of template DNA
    -
  - Digests
    - no enzyme
    - HindIIIHifi in Cutsmart Buffer (1 Cut)
      - 5,155 bp band in pZE:T7RNAPol
      - 3163 bp band in pZE:CAT
    - KpnI Hifi in Cutsmart Buffer (2 Cuts)
      - 2681 and 2458 bp band in pZE:T7RNAPol
      - 3163 bp band in pZE:CAT (1 cut site within T7RNAPol)
    - KpnI and HindIIIHifi in Cutsmart Buffer (3 Cuts)
      - 2490, 200, and 2469 bp bands in pZE:T7RNAPol
      - 645 and 2400 bp bands in PZE::CA
  - Run in Gel and examine for banding.


2
3000
1000
500
Double



Biofilm Assays

Use *Pseudomonas aureginosa?* Or certain *E. coli* strains.

(from Dr. Isaacs' paper) Crystal Violet assays measure cell density.
http://www.ncbi.nlm.nih.gov/pubmed/21307833 Microtiter dish assays measure EARLY stage biofilm formation, which may be what we're interested in. (Link has protocol)

- look up crystal violet assays for E.Coli and P.A. ☑
- MBEC assay for P.A. time requirement ☑
- Adv/Dis
- what kind of data do you get out ☑
- specific reagents (crystal violet dye

1) MBEC Assay- Highthroughput test that will show effect of antimicrobial agents
http://www.innovotech.ca/MBEC_HTPInstructions_Rev1.pdf

- Dilute biofilm strain in an overnight culture with LB
- Add this to a 96-well micro titer plate
- Place lid with 96 pegs on top
- Do a serial dilution and then spot plate
- Put plate on a rotary shaker for 16-18 hrs
- Biofilm growth check after incubation:
    - remove peg from lid and put into well of 96 micro tier plate containing recovery media
    - Recovery plate is sonicated
    - Cell density determined by spot plating or nano drop?
- Preparing Challenge Plate
    - will contain our antimicrobial peptide
- Rinse Plate:
    - Add buffer to 96 well plate
    - Transfer pegged lid from rinse plate to challenge plate
    - then transfer pegged lid to recovery plate with neutralizer
    - sonicate to remove biofilm
    - Spot plate
    - count colonies
    - Determine MBEC break point value: minimum amount of antibiotic that inhibits biofilm growth
    - how well DOPA binds
    - using AFM to know how much binds to plate
- Time requirement: takes about a day to do assay, and then grow overnight and do MIC and MBEC test the next day
- Data output: MIC, and MBEC, and microscopy
- Confocal Microscopy as Ben suggests:
    - fix biofilms to surface of the pegs
    - break the pegs and rinse
    - stain with appropriate fluorophores and examine with CLSM

2) Crystal Violet Staining- easiest

- Add peptide in media to 96-well plate
- Add crystal violet to stain biofilm
- Wells are photographed
    - measure crystal violet growth to measure growth of our peptide
- Prepare overnight culture of **Pseudomonas aeruginosa**
    - Transfer to a 96 well plate
    - Incubate
    - Add crystal violet to stain and then photograph
    - quantify with acetic acid
    - Read with a plate reader
    - Add in peptide and measure the values gain

- Crystal Violet Assay with P.A. http://iai.asm.org/content/73/6/3693.full
    - grow biofilm with polypropylene tubes
    - grow the biofilm strain overnight
    - sonicate the P.A. in LB
    - serial dilute and then plate on *Pseudomonas* isolation agar
    - quantify *P. aeruginosa* biofilm density with CV (crystal violet) staining
    - Incubate the antimicrobial peptide culture in a 96-well micro titer plate overnight at room temperature
    - Block all wells with 1% bovine serum albumin for 1h before adding in the P.A.
    - Label the P.A. with some fluorescent tag (onjugate carboxy-fluorescein dictate)
    - Add P.A. to wells containing antimicrobial peptide
    - Incubate plate and then wash with PBS
    - Measure biofilm density with CV staining

Crystal Violet Assays with E.Coli:

3) Inhibiting Quorum Sensing (this is a later typeof assay)

- Grow Pseudomonas aeruginosa in culture
- Suspend in sterile LB broth and add in antimicrobial peptide
- Add to a 96 well plate
- Incubate
- Use a plate reader to read absorbance to measure bacterial growth
- Remove bacteria with PBS
- Accounting for genomic DNA: "Cells and RNA were harvested using the RNA protect solution and RNeasy mini kit with the RNase-Free DNase Set to remove genomic DNA (Qiagen)"

4) Confocal Microscopy

***In Vitro* Biofilm Formation**

1. Bacterial colonies (which have been grown on appropriate agar overnight), were suspended in media and the OD_{490} was adjusted to 0.65
2. The resulting bacterial suspension was then diluted 1:6 (1 mL bacterial suspension + 5 mL pre-warmed medium) and incubated at 37°C with 5% CO_2 for approximately 3 hours in order to reach mid-log phase.
3. Dilute the mid-log growth suspension 1:2500 with pre-warmed media and place 200 µL of dilution into each well of an 8-well chamber slide.
4. Incubate at 37°C with 5% CO_2.
5. After approximately 16 hours, aspirate medium from corner of each chamber and add 200 µL fresh, pre-warmed medium - dispense along wall of chamber so as not to create shear forces within the chamber which could disrupt the biofilm.
6. If incubating longer than 24 hrs, change medium every 12 hrs or as needed to maintain bacterial viability.

2. Visualization of Biofilm

1. Aspirate medium from each chamber, as described above, and wash resident biofilm twice gently with sterile saline.
2. Add 200 µL of BacLight Live/Dead stain (3 µL component A + 3 µL component B per mL of saline) to each well and incubate at room temperature for 15 minutes. Protect culture from light from this point onward.
3. Aspirate the stain and wash twice gently with sterile saline as before.
4. Add 200 µL of neutral buffered formalin to each well and incubate for 30 minutes at room temperature to fix specimen.
5. Remove fixative and properly dispose of in accordance with institutional guidelines.
6. Wash twice with saline and dispose of the wash fluids that contain formalin as above.
7. Remove the plastic wells from the slide, add enough saline to each well so that when a coverslip is placed on the gasket, no air bubbles are present.
8. Coverslip and seal the edges of the coverslip with mounting medium. Nail polish can be used to seal the slides however the slides will not be as permanent.
9. Allow the sealant to dry for approximately 1 hour prior to examining via microscopy.

White Board

Map
Author: Ariel Hernandez-Leyva

NUMBERS
PTAEO: 1069695.00.0001BE.613001
stockroom: 505222
Keck: AHAFI843
Sequencing: D2HU
YaleiGEM: ATTGGGGAGATG

Lab Notebook: Interlab Study

Map
Author: Ariel Hernandez-Leyva
Goal: Create 3 GFP constructs with the correct promoters/backbones, and then characterize the fluorescence.

Parts: E0240 in pSB1C3 found in iGEM registry.
Promoter primers: J23101: J23101_pSB1C3 F: tttacagctagctcagtcctaggtattatgctagc, J23101_pSB1C3
R: GCTAGCATAATACCTAGGACTGAGCTAGCTGTAAACTCTAGAAGCGGCCGCGAA
J23115: J23115_pSB1C3 F: TTTATAGCTAGCTCAGCCCTTGGTACAATGCTAGCTCACACAGGAAAGTACTAGATGC J23115_pSB1C3
R: GCTAGCATTGTACCAAGGGCTGAGCTAGCTATAAACTCTAGAAGCGGCCGCG
BL21 (non-chloramphenicol resistant)

Note: J23115 is called “promoter 2” and J23101 is called “promoter 3.” This is the opposite of what the competition calls them.

Lab Notebook:

Parts Creation: Amplified up part E0240 in plasmid pSB1C3 in BL21 (redid it because we did not make a frozen stock of the strain the first time), and isolated the plasmid. Used above primers with entire promoter sequence in overhangs to piece together the plasmid and replace the original promoter in the plasmid with the correct one.

Plasmid synthesis protocol: Run PCR using the HiFi mix in two steps, with the first step being 58˚C for 7 cycles and the second being 68˚C for 20 cycles. DPN1 digest to get rid of template, and then run Gibson assembly with 10 µL of the PCR product and 10 µL of the Gibson assembly mix. Drop dialyze for more than 2 hours, and transform into BL21 strain, let recover and plate onto chloramphenicol plates.

Frozen stocks made: 7/3/2014 of screened trials.

Circularity test: A test that went nowhere checking to see if the plasmid would seal itself after multiple rounds of PCR. Turned out the forward primer was inefficient (since it was situated partially on the promoter) and the fragment too small to be screened.

Yamini plated the Interlab study plasmids in BL21 on the Cat plates. Please check how they did tomorrow.

7/9/2014
Current status: On way to being sequenced, frozen stock made of both promoter strains.

7/10/2014
Status: sent off to sequencing!
We need to transform the fully made construct sometime...

7/14/2014
DH1alpha streaked by Natalie in Incubator upstairs. We should make liquid culture tomorrow and frozen stock the day after that.

7/16/2014
Sequences received. They're...quite a mess. Going to sequence ~40 bp more upstream this time and try again.

7/17/2014
Main issue: forgot to DPN1 digest, took in original plasmid instead of new one.

Linker Research

- make sure there is a unique sequence (maybe 8-10 bases)
- create via single stranded DNA

1) Rigid versus Flexible

http://www.microbialcellfactories.com/content/12/1/25

- Linker sequences:
    - rigid linker (used in PhoC-R-GFP), EAAAKEAAAKEAAAKEAAAKEAAAK (25 amino acids)
        - Codon optimized in JCAT for E.Coli K-12 derivative: GAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGAAGC TGCTGCTAAAGAAGCTGCTGCTAAA
    - flexible linker (used in PhoC-F-GFP), GGGGSGGGGSGGGGSGGGGSGGGGS (25 amino acids)
        - Codon optimized in JCAT for E.Coli K-12 derivative:
        - GGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGG TGGTGGTTCTGGTGGTGGTGGTTCT
- PhoC-R-GFP – for rigid linker
    - Forms a rigid a-helix conformation when expressed
    - Improved inclusion body activities
- Effect of linker more important in inclusion bodies than in soluble proteins
- For soluble proteins, change of linker shows less statistical significance
- Linkers have more role in controlling conformation of fusion proteins (such as distance and orientation between domains and folding domains)
- The linker can modulate the distance between the two domains, and affects aggregation of GFP moieties
- Linker affects folding, exposing the hydrophobic parts, for example

Table 2

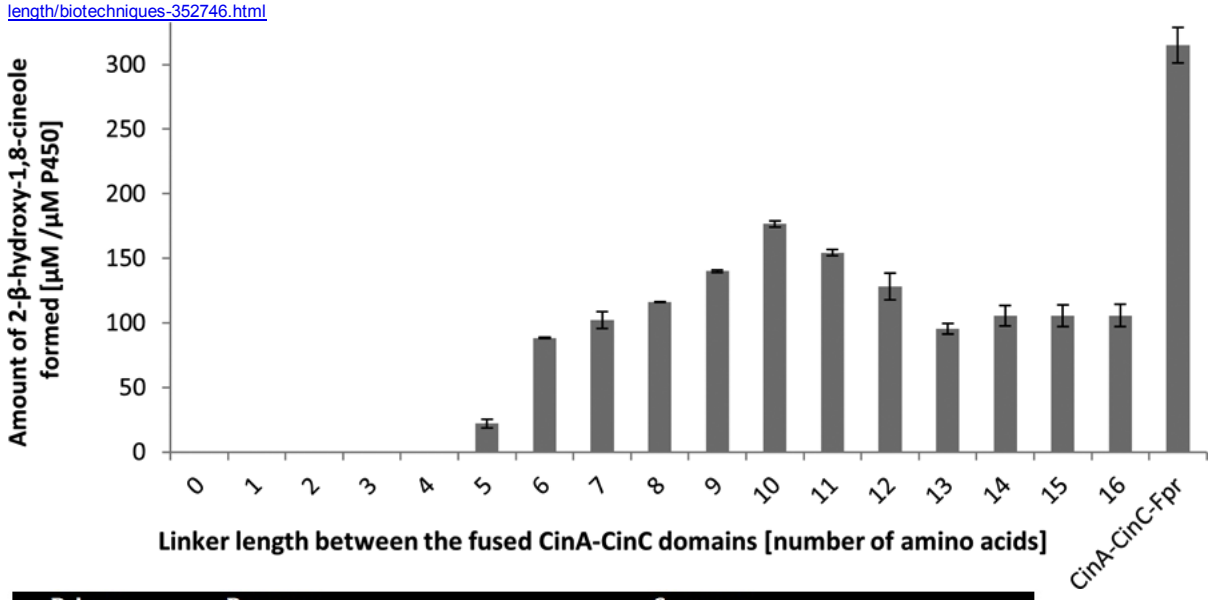
Comparison of specific activities of PhoC-GFP fusions with different linkers

Activity	Relative specific activity of IBs1 (%)			Difference in specific activity caused by linker3 (%)	
	PhoC-F-GFP	PhoC-R-GFP	Increase2	Soluble	IBs
Fluorescence	14.2 ± 0.1	22.0 ± 0.2	54.9	15.3 (5.70×10^{-5})[4]	30.7 (1.76×10^{-8})
Phosphatase	47.9 ± 1.6	58.0 ± 1.5	21.1	8.74 (1.25×10^{-1})	10.4 (6.36×10^{-3})
Phosphotransferase	62.8 ± 0.1	68.0 ± 1.3	8.28	1.61 (7.47×10^{-1})	10.0 (1.15×10^{-3})

[1] Calculated as the percentage of the specific activity of IBs relative to the corresponding specific activity of the soluble protein.
[2] Increase of relative specific activity of PhoC-R-GFP compared with PhoC-F-GFP as expressed by the following

equation: (RSA, relative specific activity).
[3] Calculated as (SA, specific activity).
[4] *p*-values for the statistical significance between different linkers regarding specific fluorescence or activity are shown in parentheses ($\alpha = 0.05$).
Huang *et al.*
Huang *et al. Microbial Cell Factories* 2013 **12**:25 doi:10.1186/1475-2859-12-25

2) Linker Length
http://www.biotechniques.com/BiotechniquesJournal/2014/July/P-LinK-A-method-for-generating-multicomponent-cytochrome-P450-fusions-with-variable-linker-length/biotechniques-352746.html



Primer Name	Purpose	Sequence
FA1	*cinA* amplification	gactcactatagGGGAATTGTGAGCGG
RA1	*cinA* amplification	**ttcgctcagacg**TTTGCCTTTCGG
FC1	*cinC* amplification	**cgtctgagcgaa**ATGGGTAATGCCCTGATTTTATATGGC
RC1	*cinC* amplification	**cgggctttgttag**CAGCCGGATCTCAG
FP1	Vector amplification	**ctaacaaagcccg**AAAGGAAGCTGAGTTG
RP1	Vector amplification	**ctatagtgagtc**GTATTAATTTCGAACATGTGAGC
RL1	R.P. for linkers 1-5	**ttcgctcagacg**TTTGCCTTTCGGAAAAATAATC
FL1	F.P. for Linker 1	**cgtctgagcgaa**CCTATGGGTAATGCCCTGATTTTATATGGCAC
FL2	F.P. for Linker 2	**cgtctgagcgaa**CCTTCTATGGGTAATGCCCTGATTTTATATGGCAC
FL3	F.P. for Linker 3	**cgtctgagcgaa**CCTTCTCCAATGGGTAATGCCCTGATTTTATATGGCAC
FL4	F.P. for Linker 4	**cgtctgagcgaa**CCTTCTCCATCTATGGGTAATGCCCTGATTTTATATGGC
FL5	F.P. for Linker 5	**cgtctgagcgaa**CCTTCTCCATCTACTATGGGTAATGCCCTGATTTTATATGGC
RL2	R.P. for linkers 6-11	**agtagatggaga**AGGTTCGCTCAGACGTTTGCCTTTC
FL6	F.P. for Linker 6	**tctccatctact**GACATGGGTAATGCCCTGATTTTATATGGCAC
FL7	F.P. for Linker 7	**tctccatctact**GACCAAATGGGTAATGCCCTGATTTTATATGGCAC
FL8	F.P. for Linker 8	**tctccatctact**GACCAATCCATGGGTAATGCCCTGATTTTATATGGCAC
FL9	F.P. for Linker 9	**tctccatctact**GACCAATCCCCTATGGGTAATGCCCTGATTTTATATGGCAC
FL10	F.P. for Linker 10	**tctccatctact**GACCAATCCCCTTCTATGGGTAATGCCCTGATTTTATATG
FL11	F.P. for Linker 11	**tctccatctact**GACCAATCCCCTTCTACTATGGGTAATGCCCTGATTTTATATG
RL3	R.P. for linkers 12-16	**agtagaaggggga**TTGGTCAGTAGATGGAGAAGGTTCGCTCAGACGTTTGCC
FL12	F.P. for Linker 12	**tccccttctact**GGAATGGGTAATGCCCTGATTTTATATGGCAC
FL13	F.P. for Linker 13	**tccccttctact**GGAGACATGGGTAATGCCCTGATTTTATATGGCAC
FL14	F.P. for Linker 14	**tccccttctact**GGAGACGCTATGGGTAATGCCCTGATTTTATATGGCAC
FL15	F.P. for Linker 15	**tccccttctact**GGAGACGCTGTTATGGGTAATGCCCTGATTTTATATG
FL16	F.P. for Linker 16	**tccccttctact**GGAGACGCTGTTGCTATGGGTAATGCCCTGATTTTATATG

*Underlined bold letters represents phosphorothioated nucleotides

- confused about why control did the best.
- 10-15 is a good number

http://download.springer.com/static/pdf/554/art%253A10.1007%252Fs00438-014-0878-5.pdf?auth66=1405714669_892253995706d830cdba2a5dd5b5ab7a&ext=.pdf

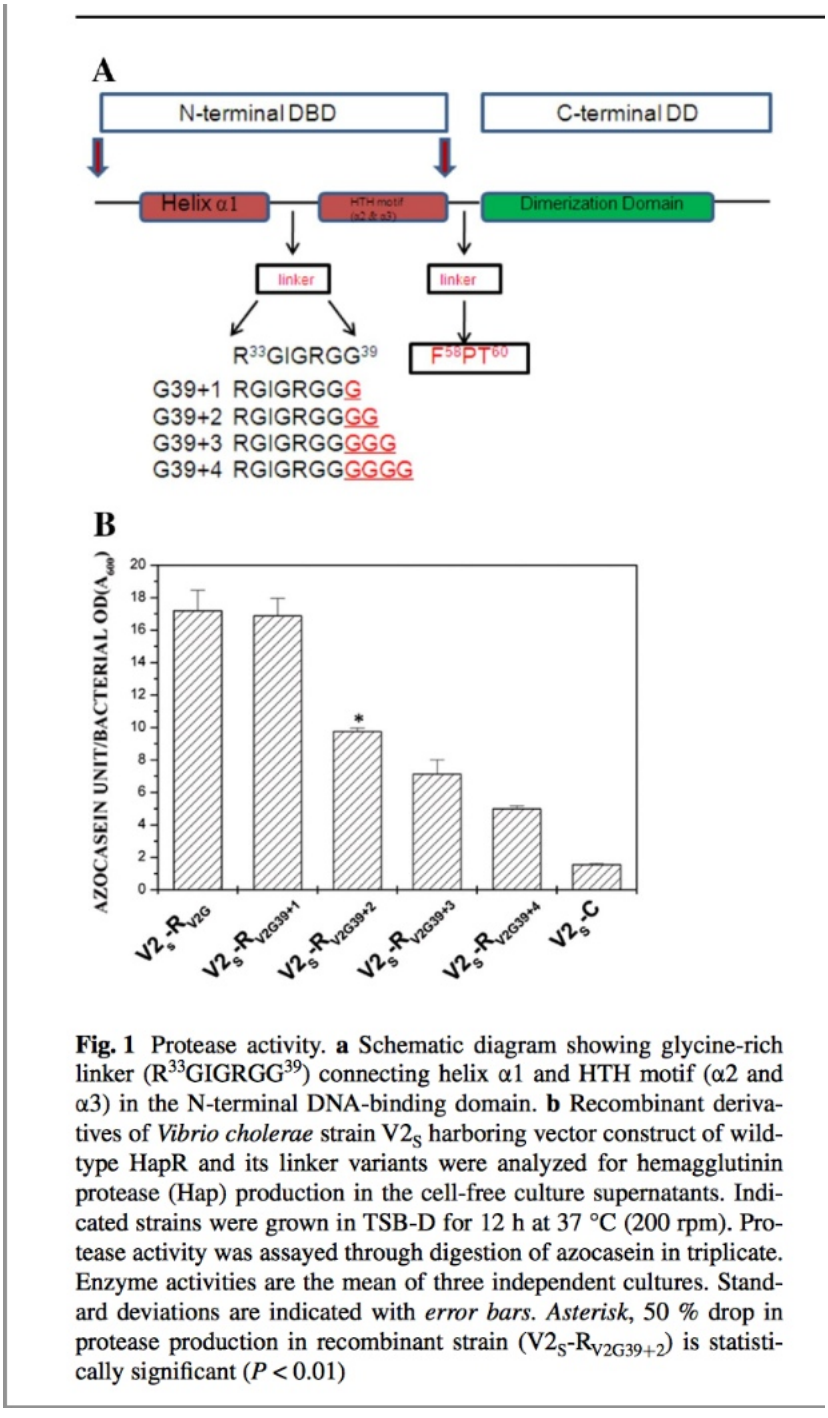


Fig. 1 Protease activity. **a** Schematic diagram showing glycine-rich linker ($R^{33}GIGRGG^{39}$) connecting helix α1 and HTH motif (α2 and α3) in the N-terminal DNA-binding domain. **b** Recombinant derivatives of *Vibrio cholerae* strain $V2_S$ harboring vector construct of wild-type HapR and its linker variants were analyzed for hemagglutinin protease (Hap) production in the cell-free culture supernatants. Indicated strains were grown in TSB-D for 12 h at 37 °C (200 rpm). Protease activity was assayed through digestion of azocasein in triplicate. Enzyme activities are the mean of three independent cultures. Standard deviations are indicated with *error bars*. *Asterisk*, 50 % drop in protease production in recombinant strain ($V2_S$-$R_{V2G39+2}$) is statistically significant ($P < 0.01$)

- Adding more glycines doesn't seem to help, but we are adding serine, glycine, not adding glycine sequentially

Suggestions from Meeting with Dr. Isaacs

- Ask Jaymin for preliminary data on control selections and viability for backbone ☐
- Get 5% DMSO from Jaymin for Mini-Prep ☑
- Ask Jaymin for MOCK-1 ☑
- Write up new protocols and show Natalie to make sure we have all the updates ☐
- Try out our 4 clones of the T7 RNA pol + pZE21 at 58 degrees annealing temp again ☑
- Obtain T7-GFP construct from Jaymin ☑
- Look at different pairs of primes so that we are convinced we have the T7 RNA pol and then submit for sequencing ☐
- Make sure we do another cloning this week ☐
- Borrow plates from someone else to test transformations against ours ☐

- Run gels for longer than 30 minutes for better separation
- We need to choose wider ranges for our temperature gradients. Decrease resolution and increase the region of sampling. Sampling every 2 to 3 degrees.
- Drop annealing temperature of primers to 56 or 57
- When we have a long gel, we need to put the ladder in between every set
- Mini-Prep: After the post-ethanol spin, put it in the incubator so the ethanol can evaporate so we get better yield and purity
- We might want to take the Mini-Preps we have and repeat the reaction with these steps and see if we get a better result
- Put cuvettes in ice and wipe them down with ice before electroplating
- Write down electroporation time constants
- When plating, use our own plates and someone else's plates
- After autoclaving the agar for plates, let it sit in hot bath for 30-40 minutes. It should not be hot to the hand when we pour the plates.
- Go with commercial primer for T7 promoter
- Annealing temperature should always be 4 or 5 degrees below melting temperature of primer with lowest melting temperature.
- Generally, we don't need to use HiFi
- If we see an arc during transformation process, tell someone immediately. This can be determined if time constant is under 4.
- From Gibson Assembly, drop dialyze 10 uL of the Gibson-Assembled product
- Streak with negative and positive controls
- Always put lid on plate for drop dialysis

General:

- Always run our protocols by others in the lab
- 30-60 seconds/kilobase for HiFi for PCR
- 15-30 seconds/kilobase for Kappa 2G Fast for PCR

7/16/14

- Do Restriction Digest
- Look into Flag Tag Purification. (if we find something we want to work with flag Dr. Isaacs)
- Dr. Isaacs is asking if anyone has SUMO on a plasmid. We would need SUMO protease under this condition.
- Recommends Manufacturing.
- Paper by Lutz and Bujerd = nucleic acids research paper 1997: describes the pZ plasmids.
    - in brief. 3 regions. Origin of replication, selection marker, promoter and gene. Origins are ColI1, p51A and pS41. Will tell us copy number of each of the origins of replication. All of them are greater than 10.
    - We will need a plasmid that needs something other than kanamycin as well as a different origin of replication.
    - We can make Gibson product of new origin of replication as well as a new selectable marker.
-

Antifouling Notes

Author: stephanie_mao

So there's different environments to consider with this project: (the proper term for the issue we're dealing with is "biofouling")

- Ship hulls
- Spaceships (interior) (SPACESHIP!)
- Pipes
- Hospitals (more from Yamini)

Common perpetrators of this foul crime are *thiobacilli*, *pseudomonas*, etc. Our best bet is probably some strain of *pseudomonas* (I can ask Ben for samples)

Factors to consider while choosing antimicrobial factors:

- Specificity: we'd probably want a
- Size: this is a physical limitation on the effectiveness of creating a conjugated antimicrobial. If we can't attach the protein to the L-Dopa leg, then it's pointless.
- Toxicity: BIG factor if we want to coat surfaces that people will touch or will come in contact with. For example, we'd want the factor to be relatively safe so the parts that wash off from the ship hull will not then go and kill a plankton population.

- Method: so there's multiple ways to kill bacteria: an antimicrobial protein can actively lyse a cell wall, or, there aremechanical surfacesthat actively dissuade cells from growing on surfaces.

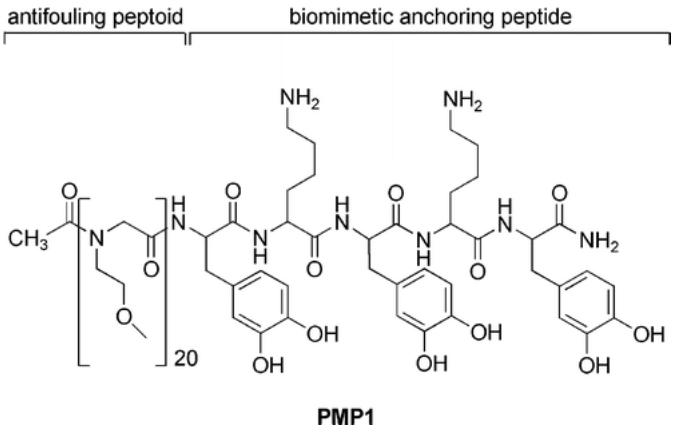
A*texture*antimicrobial factor may be preferable because it is more general and hopefully less toxic if detached from a large surface.

WELL BOY HOWDY LOOK ATTHIS HEREPAPER IT'S ALMOST AS IF IT'S TRYING TO SOLVE THE EXACT SAME PROBLEM.

Biomimetic polymers we can look into:cicada wings(other insects also have similar properties), lotus leaves, shark skins, gecko feets (wow these are all cool things! any one of these can be a mascot!)

Here'sa reviewof hydrophobic surfaces--it looks like our struggle is less about protein function and more about overall structure, which we may be able to space apart with specific designs of the protein foot.These peoplehave a more complicated idea, coating a surface with DOPA and then making the proteins*mimic*the organization of a lotus leaf. The physics of fluidity may help.

Current findings: this polypeptide-mimic compound that uses PMP1 (huh!) Unfortunately this looks like it needs post-translational modification.



PMP1

General characteristicsof antifouling consensus sequences: electrically neutral, hydrophilic, electron accepting but not electron donating.

http://davidcwhite.org/fulltext/455.pdf

Collection of links on antifouling:

http://www.bimat.org/assets/pdf/nu_05_8dalsin.pdf

http://web.mit.edu/andrew3/Public/Papers/2012/Ivanova/2012_Small_Natural%20Bactericidal%20Surfaces%20Mechanical%20Rupture_Ivanov

http://www.che.ncsu.edu/genzergroup/pubs/pub-06-11.pdf

http://pubs.acs.org/doi/abs/10.1021/ja303037j

http://science.nchc.org.tw/old_science/nano_mems/nanomidtermreport/Cicada%20Wings%20A%20Stamp%20from%20Nature%20for%20N

http://cen.acs.org/articles/90/web/2012/06/Lotus-Leaves-Mussels-Inspire-Method.html

MGFP Purification Protocol

Author: stephanie_mao

1. Recombinant mgfp-5 in *E. coli*, extracted w/ unmodified Tyr instead of DOPA
Source: http://aem.asm.org/content/70/6/3352.full.pdf (2007)

- Extracted mgfp-5 was inserted into plasmid via restriction sites, with an N-terminus His6 tag.
- Vector had Amp resistance and IPTG-activated promoter.
- Immobilized-metal affinity purification, with Ni-nitrilotriacatate NTA agarose (Qiagen) (10 ml) charged with 10 ml of 0.1 M NiSO4 (Samchun Chemicals) as the affinity purification resin.
- Pelleted cells, resuspended with 5 ml of buffer B (8 M urea, 10 mM Tris-HCl, 100 mM sodium phosphate [pH 8.0]) per g (wet weight). Centrifuged for 20

min at r.t., then collected supernatant to run on column.
- Column had 5 resin vol of Buffer B, and 10 mL of lysate was washed w/ buffer C (8 M urea, 10 mM Tris-HCl, 100 mM sodium phosphate, [pH 6.3]) and buffer D (8 M urea, 10 mM Tris-HCl, 100 mM sodium phosphate [pH 5.9]).
- Target protein was eluted with buffer E (8 M urea, 10 mM Tris-HCl, 100 mM sodium phosphate [pH 4.5]).
- Eluted protein was drop dialyzed overnight in 5% acetic acid at 4˚C using Spectra/Por molecular porous membrane tubing.
- Proteins were centrifuged at 100˚C and ran on an SDS-PAGE.
- Percent yield was 7%: future should be better.

2. Same guys as above, now with recombinant fp-151:
Source: http://www.sciencedirect.com/science/article/pii/S0142961207003651 (2007)

- "25% (v/v) acetic acid [dilution] provided a maximum recovery yield (~54%) and relatively high purity (~88%) of fl-151"

DOPA-protein amplification

Author: stephanie_mao

Using T7 pol system:

- Need to integrate T7 tact system into rE. coli genome.
- Gibson assemble construct into plasmid w/ T7 promoter.
- Transform plasmid into rE. coli strain w/ T7 tacr.
- Stimulate expression.
- Intermediary: use Jaymin's strain that also has T7 in rE. coli, but not with the tact system.

Regular system:

- Transform vendor plasmid into a good protein expression strain (non-Kan resistant) and induce expression. Should be done in conjunction with above Gibson assembly.

Construct Idea: DOPA + Spider silk

Author: stephanie_mao

Here's an idea: what if we attach spider silk in between two mussel foot proteins, and then coat a surface with it?

(Stephanie's idea)

1. Spider silk has been shown to beantimicrobial

2. Spider silk is strong and resistant to degradation.

3. Spider silk is a structural polymer and does not require terminal specificity.

4. Spider silk has been produced with a conjugated protein before bya former iGEM team.

5. Spider silk loses its stickiness over time, and its antimicrobial effects are decreased when exposed to water...DOPA can help

Spider silk is in theiGEM registry, and sequences can also be foundhere.

The idea then is to generate spider-silk DOPA peptides that, in coating a surface, creates a mesh on which biofilms cannot form. This may be a good alternative in looking at medical applications, since spider silk is NOT toxic in mammals.

Troubleshooting (T7)

Author: stephanie_mao

Okay, so we're currently having some issues with the transformations, so let's take a step back and look at what can go wrong, and how we can fix them:

Template generation: For T7 RNA pol+pZE21 with the cr/ta system, the hairpins on the primers limit the efficacy of PCR and Gibson assembly.

Solutions: First, we should screen the plasmids we have to check and see if they do in fact contain T7. Ariel has designed primers for the test already, and if necessary we can use the pZE21 with CAT as a control, since the band would be a significantly shorter length.
We can also try a different method of assembly: restriction enzyme digest (may be difficult because there are none that anneal EXACTLY to the spots we want, some are a few base pairs off), or deactivating CAT's start codon and placing T7 a little ways downstream of the crRNA sequence, INSIDE CAT (which, without the start codon, would not be translated).

Transformation: We need to drop dialyze longer. Our bacteria doesn't seem to like the high salt concentration. Do duplicate plates.
Quality control on plates: be sure to streak a non-transformed plate every time to make sure the antibiotic is working.
Transform into a hardier strain/one better suited for transformations?

Outline of Our Project

Author: Ariel Hernandez-Leyva

Goal: Create a recombinant protein using a dopamine surface anchor and an anti-fouling head-domain that can be expressed in recoded E. Coli.

- Create an expression system
    - Creating theT7 Riboregulated System
        - First, create a construct that places the gene for T7 RNA polymerase in the backbone of a plasmid with a cis-repressing and trans-

activating RNA.
- Obtain T7 RNA Pol: from team strain #5.
- Obtain pZE21 backbone: from Ryan
- Design primers for PCR and Gibson Assembly (191, 192, 193, 194, 195)
- PCR amplify T7 RNA Pol an pZE21 using primers.
- DPN1 Digest template DNA.
- Run fragments on a gel green Gel
- Gel Purification of fragments.
- Gibson Assembly of fragments into plasmid construct.
- Transform plasmid construct into K12 derivative, ECNR2. Plate on Kan Plates.
- Pick colonies from Kan Plates, inoculate liquid culture.
- From liquid culture make a frozen stock.
- Screen colonies for presence of plasmid of interest
  - First find which colonies have T7 and pZE21.
  - Use sequencing primers to amplify sequence of interest. Sequence for verification.
- Integrate into genome of ECNR2 or BL21.

- Create Promoter for T7 Riboregulated System
  - Create a construct that installs sfGFP behind promoter for T7.
    - Obtained T7 promoter from registry and Life Technologies.
    - obtained sfGFP gene from laboratory stock.
    - Approach 1: T7 Overhangs Approach
      - amplify standard pZE21::sfGFP plasmid with primers designed by Stephanie that have T7 as overhangs. Amplification of this plasmid will situate the T7 promoter in front of sfGFP.
      - DPN1 digest.
      - Gibson Assemble to circularize into plasmid.
      - Drop Dialysis to minimize salt.
      - transform into ECNR2/BL21
      - Screening for Plasmid
        - Insert plasmid into BL21 DE3 and screen for expression of sfGFP. BL21 DE3 is a strain that contains the gene for T7 RNA Polymerase.
    - Approach 2: Insert sfGFP into T7 containing plasmid.
      - Using psB1A2 with T7 promoter from registry. Transform bacteria to make stock of pSB1AC2.
      - Using primers designed by Ariel amplify pSB1AC2 to have overhangs with sfGFP.
      - Amplify sfGFP from pZE21::sfGFP with primers designed by Ariel to have overhangs with pSB1A2 with T7 Promoter.
      - DPN1 Digest
      - Gibson Assemble.
      - Drop Dialysis.
      - Transform into BL21/576
      - Screen for ampicillin/carbenicillin resistance.
      - Insert plasmid into BL21 DE3 and screen for sfGFP expression.

- Create constructs
- Express Constructs in rE.Coli
- Test Constructs using Antimicrobial Assays
- Test Constructs using Adhesion Assays.